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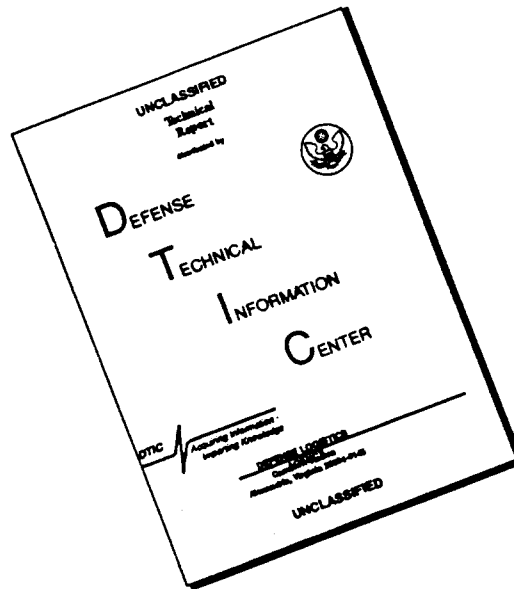
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13. ABSTRACT (Maximum 200) <p>Using a pair of murine cell lines, one lacking p53 and a derivative cell line containing temperature sensitive p53 val135 (which is wt at 32°C), we have identified the sequences in the mdm2 promoter protected by protein in the nuclear chromatin after temperature shifting. Each putative p53 response element (RE) in the ts p53 containing cell line is protected over its downstream half by 4 hours at 32°C (with further changes in protection resulting by 24 hours), while the same sequence is unprotected in the cell line without p53. Thus demonstrating dynamic p53 dependent DNA-protein interactions.</p> <p>We compared the transcription activity from three reporter plasmids containing either: 16 p53 RE's, the mdm2 promoter or the HIV-LTR in breast cancer cell lines ZR75-1 (wtp53 +/-), MDA-MB-468 (His273 p53) and MDA-MB-157 (no p53). In ZR75-1 the 16 p53 RE's showed the highest activity, suggesting that wild-type p53 in these cells can function; however DNA damaging drugs are unable to stabilize the p53 present, suggesting normal p53 activity is impaired. In MDA-MB-468 the HIV-LTR construct shows the highest activity while in MDA-MB-157 the activity of all three constructs is approximately the same. This suggests that His273 p53 in MDA-MB-468 is able to activate transcription from the HIV-LTR.</p>					
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FOREWORD

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Introduction:

We are using DNaseI sensitivity testing and *in vivo* footprinting in order to investigate if wild-type and mutant p53 bind to three potential p53 DNA binding regions in nuclear chromatin. The potential p53 binding regions we are investigating reside in 1) the mdm2 gene (40), 2) the gadd45 gene (42) and 3) the HIV-LTR (30). Because we have had difficulty establishing ts p53 Val 135 breast cell lines we began our studies using a pair of murine cell lines, one lacking p53 and a derivative cell line containing temperature sensitive p53 val135 (which is wt at 32°C).

The purpose of this work is to determine if different potential p53 DNA binding sites in nuclear chromatin are bound by different forms of wild-type p53 that may exist in normal and breast cancer cells; and to see if this DNA binding by p53 occurs at different times during the cell cycle. We are also working to determine if a direct DNA-mutant p53 protein interaction can occur in nuclear chromatin and if such an interaction is involved in the ability of p53 His273 to activate HIV-LTR driven transcription and activate latent HIV replication (16)(20) as well as activate transcription from a number of cellular genes (13)(15)(18)(71).

All previous experiments examining the DNA binding properties of p53 proteins have been carried *in vitro*, on naked DNA, with purified p53 protein produced either in bacteria or insect cells. Experiments with mammalian derived p53 proteins on the other hand have made use of crude cell extracts. We are studying the ability of wild-type and mutant p53 proteins to interact with DNA sequences (known to be bound by wild-type p53 in cell free systems) in nuclear chromatin. The status of p53 in many breast cancer cell lines has been identified, and utilizing these lines will facilitate our study as well as generate potentially useful information for the treatment of breast cancer.

Background:

p53 binds to DNA and regulates transcription

The p53 tumor suppressor protein can function as a checkpoint factor (35) causing cells exposed to DNA damaging agents to arrest in G1 (32)(41). The normal function of p53 is regulated, at least in part, by the ability of the protein to bind site-specifically to DNA (74). The tumor-derived mutant p53 proteins that have been tested thus far have altered, or deficient DNA binding activity (4)(9)(43)(44), however some oncogenic p53 mutants can bind to DNA at temperatures lower than 37°C (5)(88) or to idealized p53 binding sites (38)(87). Wild-type p53 binds nonspecifically to DNA (43) (69) as well as specifically to diverse DNA sequences that contain two adjacent copies of the consensus sequence 5'-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3' (21)(26). Wild-type but not mutant p53 can activate transcription from specific cellular regions containing p53 binding sites both *in vitro* (25) and *in vivo* (83) (25) (26). Therefore, there is a strong connection between p53 DNA binding activity and normal p53 function. Recently several genes that are induced by high levels of wild-type p53 have been identified.

Growth arrest is presumably brought about in part by specific activation of one or more of these genes. Some of the responsive genes in which p53 binding sites have been identified include the oncogene mouse double minute 2 (mdm2) (3)(40)(60), growth arrest

and DNA damage 45 (gadd45) (37)(42) (86) , and waf1/cip1 (22). The product of the waf1/cip gene is a potent cyclin dependent kinase inhibitor. This presumably accounts for the ability of wild-type p53 to arrest cell cycle progression at the G1/S border before activation of the cyclin regulated p34 cdk2 kinase (22)(33). Additionally, activation of gadd45 (which is part of the DNA damage response pathway) and mdm2 (whose protein product binds to p53 and blocks p53 mediated transactivation in cell cycle checkpoint pathways (8)) may help to signal cells to growth arrest until DNA repair has taken place. The fact that the tumor derived mutant p53 proteins do not activate transcription of the genes described above (25)(45) nor induce G1 arrest (41), may account for the gene amplification which is associated with the oncogenic state (50)(81).

In addition to promoters that are activated by wild-type p53 there are also many promoters that are repressed by wild-type p53 (15)(18) (29) (62)(70)(71). Promoter regions that fall into this category are of both viral and cellular origin. Viruses containing this class of promoter include SV40 (Alwine, personal communication; Prives et. al., unpublished; (70)), herpes simplex virus thymidine kinase and UL9 promoters (15), human cytomegalovirus-immediate-early promoter (15) and the long terminal repeat regions (LTR) of HIV, Rous sarcoma virus, and human T-cell lymphotropic virus (15) (70). Some of the cellular prototypes include proliferating cell nuclear antigen (PCNA) (15), DNA polymerase alpha (54), multi drug resistance 1 (mdr1) (13), interleukin 6 (65) as well as c-fos, p53, MHC, c-jun, β actin and Hsc70 (29). p53 can bind site specifically to SV40 DNA adjacent the SV40 origin of replication (4). This p53 binding site overlaps with binding sites for the transcription factor Sp1 and a DNA structural motif which is similar for both Sp1 and p53 binding sites has been identified (51). Although no p53 binding sites have yet been identified in the cellular genes discussed above, Sp1 binding sites are present in some and may play a part in the p53 dependent repression observed (7). Equally, if not more, intriguing is the fact that most of the viral and cellular promoters that are repressed by wild-type p53 are transcriptionally activated by mutant p53 proteins (13)(15)(18)(71). The possibility exists that in vivo both wild-type and mutant p53 can interact with promoters that contain Sp1 binding sites. In fact p53 has been shown to associate with Sp1 and this protein complex interacts with Sp1 binding sites on DNA (7).

Differential occupancy of consensus binding sites in chromatin

While some transcription factors, like GAL4 (72) and Sp1 (49), can bind specific DNA sites within a nucleosome core, others like NF1 and heat shock factor, are unable to interact with some specific DNA binding elements when the nucleosome core is assembled (1)(61). In fact it is a presupposition that in general, nucleosomes positioned over promoters are inhibitory to other proteins binding, however it has been demonstrated that this is not always the case (Reviewed in (78)). Therefore, it has been suggested that many genes are programmed during DNA replication while the nuclear chromatin assembles (79). If there are limiting transcription factors available in a cell then a gene that is replicated early in S-phase has more opportunity to assemble an active transcription complex than a gene that replicates late. This is because a gene that replicates early may be available for transcription factors to bind before all the early replicating portion of the genome has sequestered these factors. Additionally, transcriptional activators can stimulate eukaryotic DNA replication by modifying the outcome of the competition between initiator factors and histones for occupancy of the replication origin (12).

Wild-type p53 is present in low levels in normal cells (57). Therefore it may be important for p53 to belong to the class of transcription factors that organize nucleosome structures in order to help define the p53 DNA binding sites that become active enhancer elements (53). With this possibility in mind one could postulate that the p53 protein might bind to specific sites during S-phase, or that because the wild-type p53 protein has a short half life it may have to bind immediately in the presence of nucleosomes and therefore may only bind to sites that have nucleosome cores positioned in a particular way. However it is also possible that p53 binding sites exist in different chromatin states over the course of the cell cycle (as is the case for yeast replication origins which bind various transcription factors(17) (19)), and that dependent on the sequence context, p53 may choose a specific function to carry out. Additionally, if p53 is a member of the class of activators which is blocked by the presence of nucleosomes, perhaps mutation of the protein (and/or complex formation with other proteins) may confer, on p53, the ability to bind sites that the protein normally finds inaccessible. If this is the case, mutations in p53 might change the spectrum of growth-control genes that the protein activates.

Year Two with a DOD Career Development Award:

Upon completion of my first year as a new investigator I recognized, in retrospect, that my statement of work (SOW) for the current project omitted many of the tasks that had to be carried out in order to get the lab up and running. At the time that I wrote the proposal I was a postdoctoral fellow in a large laboratory that had all the necessary equipment, support personnel and lots of other researchers. When I began at Hunter College I entered empty lab space and an empty office. I had to begin from the bottom up, ordering everything from equipment to tips. Each task outlined in the SOW required many reagents to be prepared and additional tasks were required to set up conditions and establish controls. I also did not recognize that what I proposed as task #1 (which was to establish stable breast cancer cell lines expressing temperature sensitive p53 Val135) would prove to be very difficult. Through personal communication with Dr. Harvey Ozer I have learned that his lab has also had difficulty establishing some stable human cell lines with temperature sensitive p53. We have screened many possible clones and are presently screening many more. Additionally while beginning, I came to recognize that isolation of pure cell cycle fractions by centrifugal elutriation has only been accomplished using hematopoietic cell lines (47)(68)(6) and adapted suspension cultures [Hengst, 1994 #410]. Through personal communication with Dr. Andy Koff we have learned that adherent cells are very difficult to elutriate because they are sticky and therefore can only be enriched for cell cycle fractions but not well purified. In light of the knowledge gained over the past two years establishing my career, changes are proposed for the statement of work. The new statement of work follows:

Revised Statement of Work:1996

The in vivo DNA Binding Properties of Wild-type and Mutant p53 Proteins During the Course of Cell Cycle

New Task 1:

Set up a new laboratory. Months 1-12.

- a. Order equipment and reagents to supply an empty laboratory.
- b. Recruit and train four students.
- c. Begin tissue culture facility operation and characterizing breast cancer cell lines growth conditions.
- d. Set up DNaseI sensitivity and *in vivo* footprinting conditions.
- e. Purification of p53 from baculovirus infected insect cells.

New Task 2 = revision of old task 1:

Establish stable breast cancer cell lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line. Months 7-36:

- a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test ts p53 effects by Northern Blotting.
- b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.
- c. Levels of p53 in the established breast cell lines will be examined both at the p53 permissive and restrictive temperatures. Test ts p53 effects by transient transfection.

New Task 3 = revised task 2:

Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA. Months 12-36:

- a. The p53 responsive regions of the MDM-2 and GADD45 genes will be obtained from laboratories that have published the clones.
- b. Gel shift analysis of p53 binding elements with nuclear and cytoplasmic extracts.
- c. Southern blot using the using the murine ts p53val135 cell line to set up the system and test chromatin structure of the gene.

d. Footprinting of mdm-2 and gadd45 binding sequences in chromatin and with immunopurified p53.

e. In vivo footprinting of the MDM-2 and GADD45 p53 binding regions will be carried out in breast cell lines with and without ts-p53val135, and also on the cell lines treated with chemotherapeutic agents.

New Task 4 = revised old task 3:

In vivo DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines. Months 12-24.

a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

b. Intranuclear footprinting on the synchronous populations of cells will be carried out.

New Task 5: This task addresses a similar to question to that of the old task 4, however we will focus on one potential binding site for mutant p53 rather than searching for many. Months 20-48.

Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

b. Footprinting of the HIV-LTR region in the nuclei of breast cell lines containing different status p53 protein in both the absence and presence of chemotherapeutic drug treatment.

c. Comparison of the proteins from different status p53 breast cell extract bound to the HIV-LTR region.

Task 6 = old task 5:

Studies on the affect of p53 on the DNA replication of the double minute chromosomes in the breast cancer cell line MDA-MB 361. Months 12-48.

a. Examine the level of MDM-2 gene amplification level in MDA-MB 361 cell lines that express ts-p53val135, both at the permissive and restrictive temperatures.

b. Make and analyze MDA-MB 361 cell fusions with normal breast cells.

c. Carry out in vivo footprinting on synchronous populations of the above cell types.

Materials and Methods:

Growth Curve:

Plate 12, 60mm dishes with 1×10^5 cells per plate. Let cells grow ON. On day one count 2 plates from 37°C and shift 6 plates to 31.5°C. On days 2 and 3 count 2 plates taken from 37°C. On days 3, 5 and 7 count 2 plates taken from 32°C. Count cells trypsinized in 1ml of trypsin, in this way the cells counted from the. Make a semi-log plot of the data.

Isolation of Protein from mammalian cell culture lines:

Whole cell lysates: Wash 100mm plate 2X with ice cold PBS. Extraction of cells on a 150mm dish will be lysed with 2ml of whole cell lysis buffer: (0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 50mM NaCl, 25mM Tris.HCl (pH 7.5), 1mM PMSF, 50ug/ml aprotinin, 50uM leupeptin) leave for 15 min on ice. Scrape cells and put into tube. Lysates should be spun at 15,000 rpm for 15 min., and the supernatant saved.

Making Nuclear Extract: adapted from Cell 66 305-315 1991.

Wash cells 2X with cold PBS. Add 2 ml of Lysis Buffer per Plate. Spin 2300 rpm for 5 min. - (Save the supernatant for cytoplasmic extract). Resuspend the pellet in 1.5 ml of nuclear extraction buffer. Transfer to an eppendorf tube and rock at 4°C for 60 min. Spin 10 min. in microfuge in cold room. **Lysis buffer stock:** 20mM Hepes, pH 7.5, 20% Glycerol, 10mM NaCl, 1.5mM Mg Cl₂, 0.2mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 50ug/ml aprotinin, 50uM leupeptin. **Lysis Buffer:** 8.8ml Stock and 6.2ml water. **Extraction Buffer:** 1.47 ml 5M NaCl, 8.8 ml of Stock, and 4.7 ml of water.

Immunoprecipitation from cell extracts and Western Blot Analysis:

Normalize for 50-60 mg total protein of whole cell extract before begin. Add 30-40ul of 50% beads coupled to PAb421 to the extract. Rock 2-20 hours at 4°C in the cold room. Spin 5 minutes in the cold room. Wash beads 4X with 1ml of RIPA buffer (150 mM NaCl₂, 50 mM Tris pH 7.2, 1% NP 40, 2% Na deoxycholate and 0.1% SDS). The beads were resuspended in 1X protein sample buffer and boiled. Samples were electrophoresed on a 15% SDS-PAGE and electrotransferred to nitrocellulose. The blot was probed a mixture of p53 monoclonal antibodies PAb240, PAb1801 and PAb421 and the signal was visualized after incubation with goat anti-mouse second antibody by developing with ECL solutions (Amersham).

Electroporation of Breast Cell Lines:

MDA-MB-157 and MDA-MB-468 cells are grown in DMEM with 10% fetal calf serum. ZR75-1 cells are grown in RPMI 1640 with 10% fetal calf serum. Cells were washed in medium without serum and harvested with trypsin. Electroporation at .23kV was carried out with 0.5×10^6 cells/pulse in medium without serum. The amount of DNA was varied from 1 to 20ug and selection for the different cell lines was carried out at the lowest G418 concentration determined to kill all the cells of a control un-electroporated plate.

Electrophoretic Mobility Shift Assay (EMSA):

Nuclear extracts were prepared as described above. Nuclear extract (3-10ug) was incubated with 150 fmoles of the indicated p53 binding site oligonucleotide, 500ng of polydI.dC and 1ug of BSA for 30 minutes at room temperature. DNA binding reactions were carried in a 30ul volume with a final reaction buffer concentration of 20mM Hepes pH 7.5, 83.3 mM NaCl, 0.12 mM EDTA, 11.7% glycerol, 1.9mM MgCl₂, 2mM spermidine, 0.67mM DTT. p53 specific antibody (1-2ug) was added to the reactions where indicated. Samples were resolved

Extraction of Nuclei and DNaseI Treatment and *in vivo* Footprinting:

This protocol was adapted from (53) Cells were grown on 150mm plates to no more than 80% confluence before shifting the temperature using 10 plates per analysis sample. Wash plates with ice cold PBS 2X. Spin down at low speed at 4°C (2500 rpm in SS34 rotor) for 10 min. Resuspend in 2ml of RSB with PMSF. Homogenize 20 strokes and check for trypan blue exclusion. Spin down at 4000 rpm 4°C, 10 min.

Wash nuclei in 2ml RSB with out PMSF 1X. Resuspend in any where from 1ml to 4ml of RSB w/o PMSF (make the lowest # of nuclei in 1ml for 4 tubes at 250ul per tube the rest to accordingly making sure to normalize for number of cells i.e.. estimate number of cells from confluence and count in hemocytometer). Set up reactions for DNaseI treatment.

RSB: 10mM Tris pH7.4, 10mM NaCl, 3mM MgCl₂ pH 7.4, 0.5% NP40, 1mM PMSF or absent. Treat with DNaseI for 10 min. at 37°C, using 250ul of nuclei in 0.1mM CaCl₂. Add 250ul DNaseI stop (with out DNA) and then add proteinase K to a final concentration of 400ug/ml and digest overnight at 37°C. Extract once with phenol and 3X with chloroform.

DNaseI stop: 2M NH₄ OAc, 100mM EDTA, 0.2% SDS. **Southern Blot Analysis** was carried out using the standard technique described in Protocols of Molecular Biology.

Ligation Mediated PCR was carried on the DNA samples as described by Mueller and Wold (55)

Centrifugal Elutriation of ML-1 and MANCA cells:

Centrifugal elutriation was carried as previously described for the MANCA cell line (47)(68). Elutriation of ML-1 cells has not been published, but the conditions were similar to those used for MANCA cells with the exception that the cells took a little longer to pump out because they appear to be larger.

Results:

Task #2: Establish stable breast cancer lines expressing temperature sensitive p53 (ts-p53val135) while working with a murine established ts-p53val135 line. Months 7-36.

Task #2 a. Examine p53 level and sub-cellular localization in the established murine cell line at both the p53 permissive and restrictive temperatures. Test p53 effects by Northern Blotting.

Although we have not yet identified a stable ts-p53val135 expressing breast cancer cell line, we have established a ts-p53 val135 mouse embryo fibroblast cell line from the parent line 10(1) that contains no endogenous p53 protein. This line is analogous to the cell line 10.1Val5 (36)), which expresses a temperature-sensitive mutant p53 protein (codon 135, Ala to Val change) driven by an LTR promoter. 10(1) cells contain no endogenous p53 because of a deletion of the p53 gene. We call our 10(1)-ts-p53 cell line 3-4, and generated it via CaPO₄ cotransfection of 10-1 cells with the same temperature-sensitive mutant p53Val135 containing plasmid along with a plasmid encoding neomycin resistance, and stable colonies were selected in medium containing 400ug/ml of G418. The colonies were initially characterized for their ability to growth arrest at 32°C and there ability to contain high levels of p53 protein.

1. Temperature-sensitive p53 and the growth arrest ability.

To analyze the growth arrest mediated by p53, cell-cycle analysis was performed by a fluorescence-activated cell sorter (FACS). 10(1) and 3-4 cells were plated at 37°C and shifted to 32°C to induce p53 into the wild-type conformation. From day 1 through day 7, cells were harvested and fixed with 0.1% sodium azide. After treating with RNase and staining with propidium iodide, the DNA content was measured directly by cytometry. (Fig.1a)

At 37°C both 10(1) and 3-4 cell lines grew well with the S phase population of cells from 10(1) cells being 49.2% and 3-4 being 57.5%. After shifting to 32°C for 24 hours the 3(4) cells showed a significant decrease in the S phase population, dropping to 12.8%, with an increase in both the G1 and G2/M population to 31.9% and 55.3% respectively. The growth arrest was maintained from day 2 to day 7, but interestingly it was predominately in the G2/M population increasing up to 60% by day 7. For the 10(1) cells, there was no obvious difference from day 1 to day 7, with an average G2/M population of approximately of 10%.

The growth curve for 10(1) and 3-4 cell lines at 37°C and 32°C was also determined. Cells were plated at the density of 5×10^4 cells per plate (60mm) at 37°C. One set continued growing at 37°C, the other set was shifted to 32°C on second day. The cell number was counted every day. 3-4 cells grow in exponential rate at 37°C but their growth is efficiently inhibited at 32°C.(Fig. 1b). 10(1) cells grow well at both 37°C and 32°C (data not shown). Because there was no obvious increase of 3(4) cell number at 32°C, the G2/M FACS analysis data clearly demonstrate that most cells arrest in the G2/M phases. These results indicated that ts p53Val135 at 32°C arrests cell cycle at the G2 phase after

the cells complete DNA synthesis to prevent further cell division in addition to blocking G1 progression into S. Interestingly, ts p53Val135 in 3-4 cells at 37°C have an aberrant cell cycle distribution (with a high portion in S phase) as compared to the 10(1) cells.

2. Nuclear localization of wild-type p53 and mutant p53.

It has been reported that by that by immunostaining technique, ts p53Val135 protein is predominantly in the cytoplasm at 37°C, but at 32°C it is translocated to the nucleus (52). The transportation of p53 from the cytoplasm to the nucleus does not require protein synthesis. Since we are studying the DNA binding ability of p53, which occurs in the nucleus, we used immunoprecipitation and Western blotting technique to check the protein level of p53 in both the nucleus and cytoplasm of the 3-4 cell line at different conditions. 3-4 cells were plated at 37°C until 80% confluence, then shifted either to 39°C for 18 hours, or to 32°C for 2 hours, 4 hours, 24 hours. After incubation, the cells were lysed to obtain the nuclear and cytosolic cell extract. The same amount (150ug) of nuclear or cytosolic protein was immunoprecipitated by antibody 421 linked to protein A sepharose. Antibody 421 can recognize both wild-type and mutant p53. The bound proteins can be eluted by heat, then detected in Western blot. (Fig. 2b).

The results show that at 37°C and 39°C when p53 is in the mutant conformation, the same portion of p53 is found in cytoplasm and nucleus. After shifting to 32°C, at which p53 is in the wild-type conformation, the portion of p53 in nucleus increases as early as 2 hours after shifting and increases further by 4 hours. (Fig. 2a and Fig 2b). But the total amount (nuclear plus cytoplasm) of p53 protein does not have a significant change, the change is only predominantly in the localization of p53 (Fig. 2c).

3. Transactivation of mdm-2 by wild-type p53 and mutant p53

To confirm the transcriptional activity of p53 in 3-4 cells and set up experimental conditions for the following DNA binding assay, the mRNA level of mdm-2 was checked by Northern blot. 10(1) and 3-4 cells were plated at 37°C and then shifted to 32°C for 4 hours. Total RNA was extracted and poly-A mRNA was purified by an oligo-dT column. 1.8ug of mRNA from all sample except 10(1) 32°C (for which less RNA was obtained, so only 1ug was loaded) were loaded onto an agarose gel and transferred to positively-charged nylon membrane. The membrane was probed with [³²P]radioactive labeled mouse genomic mdm-2 Apa I-Nsi I fragment extracted from the genomic clone in the plasmid 3A-5 (kindly provided by Dr. Donna George), which contains part of exon 1 and the entire exon 2 of mdm-2. This data was quantitated using a phosphorimager.

At 37°C both 10(1) and 3-4 showed the same basal level of mdm-2 expression, but after incubation at 32°C for 4 hours an increased amount of mdm2 mRNA of 2.6 fold was seen in the 3-4 cell line (Fig. 3a). Although the mdm2 message in the 10(1) cell line at 32°C appeared to go down, probing the same membrane with a [³²P] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) house keeping gene probe demonstrated that less RNA was loaded for that sample (Fig 3b) however, the mRNA level of GAPDH in the 3-4 cell line at both temperatures was the same. This indicates that the increased transcription

of the mdm-2 gene seen at 32°C for 4 hours was in fact p53 dependent (compare Fig. 3a to Fig. 3b).

Task #2 b. Attempts will be made to establish ts-p53val135 expressing breast cancer cell lines of MDA-MB 468, ZR 75, MDA-MB 157, MDA-MB 361 and MCF10A.

The temperature sensitive characteristics for the ts-p53 val135 plasmid construct have been shown to behave in the same manner in human cells (82), therefore I assumed that screening the stable breast cancer cell lines with the ts-p53val135 selected for G418 resistance could be done as described above for the murine cell line. Unfortunately, the effect of a temperature shift down on the breast cancer cell lines MDA-MB-468 (containing p53 His273), ZR 75-1 (containing wild-type p53) and MDA-MB157 (containing no p53 due to deletion of the endogenous gene) revealed severe growth retardation at 32°C (data was shown in the last progress report). All three cell lines can grow if shifted back to 37°C demonstrating that the cells are still alive, thus we concluded that we could continue trying to make the stable cell lines however screening for growth retardation can not be a primary screen for the G418 selected colonies. Therefore, we must test all G418 selected colonies for the level of p53 and subcellular localization in order to score for positive colonies.

Selected stable transfected colonies of MDA-MB 468 with p53 Val135 are presently being selected. 1 x 10⁷ cells were transfected by electroporation at 350V with various amounts of DNA and selected with 200 mg/ml of G418. A total of 40 different clones were picked from two independent transfections with 10 mg of supercoiled plasmid encoding resistant to Neomycin and 10 mg of supercoiled plasmid containing the temperature sensitive p53 Val 135 gene. 10 clones were picked from cells transfected with 10 mg of supercoiled plasmid encoding resistant to Neomycin only. 2 clones were picked from cells transfected with 10 mg of Neomycin and 10 mg of p53 Val 135 linearized DNA. One clone was picked from cells transfected with 10 mg of Neomycin linearized DNA only.

Task #2 c: Levels of p53 in the established cell lines will be examined both at the p53 permissive and restrictive temperatures.

Levels of p53 Protein in Breast Cancer Cell Lines MDA-MB-468, MDA-MB-157 and ZR75-1.

We chose to begin growing three breast cancer lines with different p53 status; one producing wild-type p53 protein (ZR75-1), one producing mutant p53 protein (MDA-MB-468) and one producing no p53 protein due to a gene deletion (MDA-MB-157). We are interested in examining the p53/DNA interactions at the mdm2 responsive region, therefore it is helpful that the level of mdm2 mRNA in two of the above cell lines has been reported (67). Although the mdm2 mRNA level for MDA-MB-157 has not been documented, the expression of mdm-2 in ZR75-1 is known to be very high while no detectable mdm-2 mRNA has been observed in MDA-MB-468. Therefore it is presumed that the wild-type p53 in ZR75-1 is activating the mdm2 gene while the mutant protein in MDA-MB-468 is not.

We are in the process of screening breast cell lines selected for G418 resistance. The levels of p53 in the parent lines should be increased if a colony is expressing the ts p53Val135. As we believe it is necessary to know the a baseline levels, whole cell, nuclear and cytoplasmic extracts were prepared from the three cultured cell lines discussed above. Immunoprecipitation of p53 from the different extracts was carried out using the p53 monoclonal antibody PAb421 (derived from the growth medium of monoclonal antibody cells) coupled to protein A sepharose. Whole cell extracts from the three cell lines were normalized at a level of 1mg of total protein per immunoprecipitation and the nuclear and cytoplasmic extracts were then normalized via the number of starting cells. The immunoprecipitated protein was analyzed by SDS-PAGE and Western blotting with a mixture of p53 monoclonal antibodies PAb421, PAb240 and PAb1801 (all of which were from the growth medium of monoclonal antibody cells). The protein bands were then visualized, after second antibody treatment, with ECL reagent from Amersham. As shown in Fig. 4 (lanes 9 & 10), p53 protein was clearly evident in the nuclear as well as whole cell extracts derived from the cell line MDA-MB-468. This is not surprising as this mutant p53 protein has previously been documented at high levels in this line (75). Interestingly, we were able to visualize wild-type p53 in the nuclear extract from ZR75-1 (Fig. 4, lane 6 as indicated by the arrow on the right). We are unaware of wild-type p53 protein levels being reported previously for ZR75-1. From this detection it appears that the gene in ZR75-1 encodes an arginine at codon 72, a neutral sequence polymorphic site, which differs from the proline encoded by the mutant p53 in MDA-MB-468 (75) and results in a faster migrating form of the protein on SDS-PAGE. This is fortuitous as it allowed the low level of wild-type p53 to be visible right below the background band of heavy chain IgG at 55K (Fig. 4, lane 6). Although the antibody was cross-linked to the solid support a substantial portion was release upon boiling. No p53 protein is present in MDA-MB-157.

No G418 selected colonies have shown an increase in the level of p53 above basal expression (data not shown). Hopefully one of the colonies we are presently screening will be positive for an increased level of ts-p53 Val135.

New Task 3 = revised task 2:

Footprinting of the MDM-2 and GADD45 p53 DNA binding sites in nuclear chromatin of unelutriated cells and on naked DNA. Months 12-36:

Task #3a: The p53 responsive regions of the mdm-2 and gadd45 genes will be obtained from laboratories that have published the clones.

Genomic clones of murine mdm2, human mdm2 and human gadd45 have generously been provided by Dr. Donna George (40), Dr. Moshe Oren (85) and Dr. Albert Fornace (37).

Task #3b: Gel shift analysis with nuclear extract from 10(1) and 3-4 cell line:

We have tested the ability of the nuclear and cytoplasmic lysates shown in Fig. 2 to gel shift an oligonucleotide containing the p53 responsive region of the murine mdm2 gene. The mdm2 gene has two promoters, the first (P1) is constitutively active while the second (P2) can be activated by wt p53 via a sequence-specific interaction. Two putative p53 response elements (p53-RE's) that share limited degrees of homology with the

defined consensus sequence have been identified in the P2 region (2). A monoclonal antibody specific for p53 (PAb 421) is able to induce a p53 specific gel shift of an oligonucleotide containing both p53 RE's when added to reactions containing nuclear extract derived from the ts p53 cell line shifted to 32°C for either 2 or 4 hours (Fig 5, lanes 4 and 6), but not when added to reactions containing extract from 10(1) cells at any temperature or 3-4 cells placed at 39°C (Fig. 5, lanes 1-3, 5 & 7)). Interestingly, this shift decreases in level and size when the cells are left at 32°C for 24 hours (Fig. 5, lane 8). No p53 specific gel shift was evident in the absence of p53 specific antibody (data not shown). We have purified p53 from baculovirus infected insect cells and this purified protein was compared for its gel shift species with and without PAb421 (Fig. 5, lanes 9 and 10). The fact that the p53 gel shift present with the 3-4 extracts plus PAb421 is higher than that present with purified p53 and PAb421 (Fig. 5, compare lanes 4, 6 and 8 to lane 10) suggests that other proteins in addition to p53 and antibody are associated with the mdm2 oligonucleotide in these reactions.

Task #3c: Different DNase I sensitivity in p53 consensus sequence of mdm-2 in chromatin.

In order to elucidate how p53 activates initiation of transcription, we focused on the DNA binding of the protein to chromatin and thus carried out *in vivo* DNase I protection to assay for changes in the chromatin structure. DNase I (Deoxyribonuclease I) is a digestive enzyme that degrades DNA nonspecifically. Bound proteins will protect the phosphodiester backbone of DNA from DNase I-catalyzed hydrolysis. Inside the nucleus, the DNA binding proteins include both non-histone and histone proteins. Therefore, by using DNase I protection analysis, we were able to screen p53 binding to its consensus sequence while examining the change of other chromatin binding at the same time.

10(1) and 3-4 cells were plated at 37°C until 80% confluence, shifted either to 39°C for 18 hours, or to 32°C for 4 hours or 24 hours, then washed with prechilled PBS twice and the cells were collected by scraping. The chromatin structure does not change at 4°C, therefore the cells were kept on ice for the remaining of the procedure. Cells were lysed gently to extract nuclei with a cell homogenizer in lysis buffer containing NP-40 to extract lipids from cell membrane and PMSF (proteinase inhibitor to prevent protein degradation). DNase I can not go into nuclear membrane directly, so the nuclear membrane was permeabilized by NP-40 and the PMSF was washed off to avoid blocking DNase I activity. Nuclei were treated with increasing amounts of DNase I containing Ca^{2+} . The DNase I curve was tested before to give an even digestion ladder. The DNase I reaction was stopped by adding proteinase k and the DNA was purified by phenol/chloroform extraction. The digested DNA samples were treated with the restriction enzyme Nsi I, which can cut adjacent to the p53 binding sites in the mdm-2 gene. The Nsi I treated DNA samples were run on a 0.8% agarose gel and transferred to the nylon membrane. The membrane was probed with the same probe used in Northern blot (Apa I-Nsi I fragment). The results were visualized by auto radiography. (Fig. 6a, 6c and 6d)

The data shows that the mdm-2 sequence containing p53 consensus sites in chromatin has different sensitivity to DNase I at the different condition tested. The DNA which has not been treated with DNase I shows a single band around 4-5 kb after digestion with Nsi I. This suggests that the mdm-2 gene in these 10(1) and 3(4) cell lines is

a single copy gene. Normally in this type of DNase I sensitivity assay, genes are not being expressed are less sensitivity to DNase I than genes that are primed for expression or actually being transcribed. At 39°C, in addition to the 4-5kb band, both 10(1) and 3(4) shows another band appears at 1 kb at low levels of DNase I treatment. This means there are one or two DNase I hypersensitive sites between two Nsi I cutting sites. This whole region is heavily protected from DNase I, and this protection is not p53 dependent, since there is no difference observed between 10(1) and 3-4.(Fig.6a).

On the other hand, at 32°C for 4 hours, 10(1) cells shows more sensitivity to DNase I than 3(4) cells, 4-5 kb fragment from 10(1) cells was lost at intermediary DNase I level (2ug), while for 3(4) cells, a resistance was evident as the band at 4-5 kb remained present (Fig 6c). We know that there is no p53 dependent mdm2 transcription in the 10(1) cell line, therefore the increased sensitivity of the 10(1) DNA shows that the P2 promoter region is primed for transcription even though it is not turned on (a similar sensitivity is clear at 37°C). Another change in 3-4 chromatin is the change of DNase I hypersensitive sites. The small band is at the 500 bp position instead of 1 kb and this change is p53 dependent. (Fig. 6c). We assume this is due to p53 binding. This difference between 10(1) and 3-4 does not exist at 37°C (data not shown). At 32°C, p53 binding may cause a conformation change of chromatin and make some other point on DNA more exposed to DNase I. Usually the transcriptionally active regions of DNA shows higher sensitivity to DNase I, and our data agrees with this as the resistance of the mdm2 P2 region to DNase I was lower at 32°C for 4 hours compared to 3-4 cells at 39°C. We speculate that at 39°C the gene is no longer primed for transcription and that is why it is resistant to DNase I.

Interesting, at 32°C for 24 hours, 10(1) and 3-4 cells show no difference for DNase I sensitivity again. Both are very sensitive to DNase I, and the p53 protection band at 500 bp has disappeared and the lower hypersensitive band is back to 1 kb. (Fig. 6e) After probing with the mdm-2 probe, all the same blots were probed with the GAPDH probe, which indicated even loading and a consistent complete digestion at 8ug of DNase I for all the conditions tested (Figs. 6b, 6d and 6f)

These Southern blot data provided important clues to further our research on the DNA binding ability of p53 *in vivo*. Taken together with the gel shift data they suggest that after 24 hours at 32°C the p53 in the 3-4 cell line has either changed its interaction with the mdm2 DNA or has changed its association with other protein factors on the DNA.

Task #3d: Footprinting of mdm-2 and gadd45 binding sequences in chromatin and with immunopurified p53.

To locate the DNA binding sites of p53 *in vivo*, ligation mediated PCR footprinting technique was used to detect the real space at the nucleotide level. This technique was first described by Mueller and Wold (55). The general procedure consists of 4 steps. The DNA samples were first digested with DNase I *in vivo* as described before. Gene-specific oligonucleotide #1 was annealed to denatured genomic DNA and extended with Sequenase DNA Polymerase to make blunted end. Next, a common linker oligo with a high melting temperature was ligated to the blunt ends of genomic DNA fragments. Then the DNA fragments were amplified in PCR utilizing different sets of primers that were as follows. The two primers were gene-specific oligonucleotide #2 and one strand of linker

oligo. Finally, the amplified DNA were visualized by elongation of radiolabeled gene-specific oligonucleotide #3. The three oligonucleotides were selected by using Mac vector computer software based on the genomic sequence of mdm-2 gene. (see Fig. 7a and 7b) The primers are approximately 350 bp away from the p53 RE's, and have increasing melting temperatures to ensure their specificity. The melting temperature for oligonucleotides 1, 2 and 3 respectively are 52.3°C, 55.3°C and 60.8°C (see Fig. 7b). Prior to beginning the *in vivo* footprinting the melting temperatures of the oligonucleotides were tested at different concentrations of Mg^{2+} the plasmid DNA containing the murine mdm-2 genomic clone. All the T_m 's were approximately as expected (data not shown).

The results presenting the nuclear chromatin footprinting data of the mdm2 P2 promoter region are shown in Fig 8. At 39°C and 37°C all samples digested with DNase I shows a 10 bp cleavage ladder (which is typically a rotationally phased nucleosome) around the p53 consensus site region and there is no p53 dependent protection evident.(Fig 8a lane 1-4). This indicates that this mutant p53 which is mutated at central core region has lost the DNA binding specificity *in vivo*. More importantly is that at 32°C for 4 hours, there is an obvious difference between 10(1) and 3-4 cell lines. The two p53 putative binding sites are protected within their downstream 3' halves in the 3-4 line but no protection is present in the 10(1) line within this region. Each of the *in vivo* protected regions contains 13 bp and there are 20 bp between the two responsive elements (RE). (Fig. 8a, lane 8 marked by the top two brackets and Fig. 8b). After reading the sequence, we noted that each p53 protected site fit the PuPuPuC(A/T)(T/A)G direct contact model which was created based on the crystal structure of p53 core domain (14). This DNase I protected sequence in chromatin does not consist of the total published putative binding region. Since the 5' half of each of the two putative p53 responsive element have 2 mismatches compared to the sequences published p53 consensus sequence, p53 may not recognize the 5' halves. It is also likely that histone packaging of the DNA in certain way may make the 3' half more favorable for access by p53. In addition, the 20 bp distance between the two REs, may utilize histone packaging to bring the two sites together in such a way that they work together behave as one site. Next to each p53 binding region, there are some new DNase I cutting bands. This may be because of an increased mobility of nucleosomes after p53 binds to DNA, either by sliding to an adjacent sequence or by transferring to a different stretch of DNA, or it may be because either p53 or other proteins facilitate the removal of nucleosomes from the DNA in this region and the conformation is changed.

Another interesting site located adjacent to the p53 REs is the TATA box. It has been published that the DNA binding ability for p53 and TBP together, to an RGC-TATA containing fragment, is increased 4-fold as compared to p53 and TBP individually (10). By comparing the DNase I pattern observed in nuclear chromatin under different conditions and the DNase I pattern for naked DNA (Fig. 8a, lane 1-10 vs lane 12), the protection of TATA box region is changed in both 10(1) and 3-4 lines. In both the cell lines the TATA box region is protected perhaps by a nucleosome as is suggested by the Southern blot test for DNase I sensitivity. This may be due to TBP because TBP can recognize most TATA sequences on naked DNA with a K_d of 10^{-9} to 10^{-10} M, but is unable to bind to certain nucleosomal templates at concentration as high as 10^{-6} M (46). For 3-4 cells at 4 hours at 32°C, the TATA box region shows prolonged protection, with new hypersensitive sites in the middle the of TATA box. (Fig. 8a, compare lanes 7 and 8) This prolonged protection

region may be due to the binding of some general transcription factors recruited by p53, such as TBP along with TAFs and TFIIB. We would like to try to identify if this is the case by using DNA affinity chromatography to compare the proteins bound from various cell extracts.

The samples shifted to 32°C for 24 hours showed no protection at either p53 RE for both 10(1) and 3-4 cell lines. (Fig. 8a, lane 9 and 10). Instead, there was an extended area of protection visible only in the 3-4 sample which extended over the TATA box and the 3' adjacent region. There are two possibilities to explain this. One possibility is that the initiation of transcription by the recruitment of TFIID and the poly II holoenzyme to the mdm-2 promoter region is dependent on the transcription activator p53 and after activation the initiation complex forms a stable subcomplex on the promoter region that allows for multiple initiation events. Another possibility is that after the induction of mdm-2 by wt-p53, the MDM-2 protein can form a complex with p53 that inhibits the binding of p53 to its consensus sequence. While MDM-2 (being a potential DNA binding protein) binds to the promoter region to block the binding of general transcription factors and the extended protection may be due to a complex of MDM-2 and p53 bound to the DNA.

We have begun setting up the conditions for ligation mediated PCR footprinting of the mdm2 and gadd45 p53 binding regions in nuclear chromatin. The oligonucleotides for these procedures have been selected using the MacVector program and we are testing the true T_m's by hybridization with the plasmids containing the genomic clones (described in task 3a). Figures 9 and 10 show the oligonucleotides (and their positions in relation to the p53 binding sites) that have been selected for the human mdm2 procedure and the human gadd45 procedure.

Task #3e: Footprinting of the mdm2 and gadd45 sites in nuclear chromatin will be carried out in breast cell lines with and without ts p53Val135, and also in cell lines treated with chemotherapeutic agents.

Stabilization of Wild-type p53 in the Breast Cell Line ZR75-1.

In addition to attempting to create the ts-p53val135 breast cancer cell lines, we have decided to try various methods to stabilize the wild-type p53 present in the ZR75-1 line. Because we have not yet selected any ts p53Val135 stable breast cancer cell lines we are working to identify if we can activate the wild-type p53 protein in the cell line ZR 75-1.

The motivation for this is that if we increase the level of p53 we may be able to invoke further p53 specific mediated responses. Additionally the way in which p53 stabilization is induced (i.e. inhibition of the ubiquitin proteolysis pathway versus the induction of DNA damage evoked by many chemotherapeutic agents) may differentially influence p53 protein activity. This will give us more insight into the regulation of the *in vivo* DNA binding properties of the protein.

The levels of p53 in normal cells is induced upon DNA damage (41). Wild-type p53 levels are thought to be low in cells due to the fact that the protein is actively degraded by the ubiquitin-dependent pathway (66). Upon treatment of ZR 75-1 cells with the DNA

damaging agents actinomycin D, etoposide and camptothecin, p53 levels did not increase as dramatically as they did when cells were treated with an inhibitor of the ubiquitin-dependent proteolytic pathway, benzyloxycarbonyl-leucyl-leucyl-leucine aldehyde (2-LLL-CHO) (58). The reagent was synthesized by, and was a generous gift of, A. Vinitsky who works on inhibition of proteolytic activity (73). Comparing figures 11a and 11b demonstrates that when 5.0 μ M 2-LLL-CHO was added to the growth medium for increasing intervals of time, a dramatic increase in the level of p53 present in comparison to the levels without drug occurred after 4 and 7 hours of incubation in both nuclear and cytoplasmic extract (Fig. 11a, densitometric scanning) while for the cells treated with DNA damaging agents no substantial change was evident until 28 hours after treatment with Actinomycin D and Camptothecin (Fig. 11b). To our knowledge, this is the first time stabilization of p53 has been documented to occur in response to a ubiquitin proteolysis pathway inhibitor. The fact that the p53 is not rapidly stabilized in presence of DNA damage suggests that the pathway which allows p53 to sense DNA damage is not functioning properly in this cell line.

We tested the ability of nuclear extract prepared from the ZR 75-1 cells to induce a gel shift of different oligonucleotides containing p53 DNA binding sites (specifically the mdm2 oligo and an oligo derived from a p53 binding region of the ribosomal gene clustered called RGC). We did not see any p53 specific gel shift that could be identified by the addition of monoclonal antibodies to DNA binding reactions (Fig. 12). However EMSA experiment with ZR 75-1 drug treated extracts on the RGC oligo did show a change in the binding pattern, in the form of a shifted species reproducibly disappearing, for reactions containing extract from cells treated with Actinomycin D for 28 hours, (compare Fig. 12a to Fig 12b). We obviously need to compare these results to those for a normal breast line treated with DNA damaging agents, but it is encouraging that we see a shifted form that goes away. This suggests that we will be able to use the *in vivo* footprinting technique to monitor the changes that occur over different regions of the chromatin as result of treatment with DNA damaging drugs. However we may not see a great response in this cell line as compared to normal cell lines and this may be a means to screen new therapeutic approaches in the future.

New Task 4 = revised old task 3:

***In vivo* DNA footprinting of synchronous populations of hematopoietic and breast derived cell lines. Months 12-24.**

Task #4a. Synchronous populations of the various hematopoietic and breast cell lines will be prepared by both centrifugal elutriation and drug treatment.

The hematopoietic cell line ML-1 has been shown to dramatically stabilize its p53 protein upon DNA damage and as a result the gadd45 gene is turned on (42)(37). The fact that we knew that we could stabilize wild-type p53 in ML-1 cells along with the possibility of being able to get an excellent elutriation profile with them suggested that this cell line would be a valuable tool for investigating the a base line for the interaction of p53 with DNA binding sites in nuclear chromatin over the course of cell cycle in the presence and absence of drugs. Thus we carried out an elutriation run of this cell line (Fig. 13a) and examined the p53 levels in the cell cycle fractions in the absence of DNA damage (Fig.

13b and Fig. 13c). Interestingly we saw that the p53 levels peaked at the G1/S border (which is one of the cell cycle checkpoints for p53).

We proceeded to compare the p53 levels in three different hematopoietic cell lines in response to two DNA damaging agents, zeocin and camptothecin (Fig. 14a). As described ML-1 cells are known to increase their p53 level in response to DNA damaging agents, HL-60 cells have no p53 due to a deletion of the endogenous gene (77) and MANCA (which are a common line used for elutriation cell cycle studies) have not had their p53 status documented. As expected the p53 levels in the ML-1 cells increased from barely detectable to 14000 fold activation as determined by densitometric scanning (Fig. 14b), while no p53 was detectable in HL-60 cells. Interestingly MANCA cells begin with a high level of p53 before treatment and this p53 can be slightly increased (Fig. 14a and 14b). We are trying to determine if the p53 in the MANCA cell line is wild-type and may be affected in similar way to what is occurring in the ZR 75-1 cell line.

We have tested nuclear extract prepared from these drug treated cells for its ability to bind to gadd45 and mdm2 oligonucleotides as assayed by EMSA. No change is seen for the MANCA extracts (data not shown). However an increase in the gel shifting ability for the ML-1 extract from zeocin treated cells is increased for both mdm2 and gadd45 oligonucleotides while treatment with camptothecin causes a reduction. Zeocin a drug available from Invitrogen and it acts as a direct DNA damaging agent (similar to the action of bleomycin). These results are encouraging and we intend to carry out elutriation of ML-1 cells treated with drug and carry out *in vivo* footprinting on elutriated cell cycle fractions.

The elutriation profile of MANCA cells was also carried out (Fig. 16a) and the p53 levels in the cell cycle fractions were determined (Fig. 16b). Interestingly the p53 profile was very different, once again peaking just prior to S but not dropping after early S as was seen so dramatically for the ML-1 cell line (see Fig. 13). As the levels of p53 were very high throughout we tested the DNA binding ability of the p53 in nuclear extracts from the elutriated fractions by EMSA (Fig. 17). In this case we were able to identify a p53 specific 1801 induced super shift that corresponded directly to the levels of p53 seen during the elutriation profile (compare Fig. 17 to Fig. 16b). There are obviously many experiments that remain to be done and the preliminary results are encouraging.

New Task 5: This task addresses a similar question to that of the old task 4, however we will focus on one potential binding site for mutant p53 rather than searching for many. Months 20-48.

Test to see if mutant p53 His273 is able to bind to the HIV-LTR region when it is transiently transfected into the cell line MDA-MB-468.

Task #5a. Examine HIV-LTR driven transcription in breast cell lines containing different status p53 protein.

BACKGROUND

The wild type product of the p53 gene had been shown to be a tumor suppressor by many criteria(24). This activity of p53 is dependent, in part, on the ability of the protein to activate transcription. Transcriptional activation in turn is dependent on the ability of the

wild type protein to bind to DNA in a sequence specific manner((5)(59) (76)). Mutants of p53 isolated from human tumor cells are defective in sequence specific DNA binding and transcriptional activation of genes normally activated by wt p53 (84). These observations indicate that loss of p53 tumor suppressor activity is directly responsible for a predisposition to the development of tumors.

Early studies suggested that mutants of p53 can give cells a growth advantage not only by losing the tumor suppressor activity but also by gaining oncogenic functions such as; the ability to immortalize primary cells (39), ability to cooperate in transformation with other oncogenes(23), ability to bypass growth factor requirements for initiation of DNA synthesis (27), and an enhanced proliferation rate(18). Additionally, mutant p53 is required for maintenance of the transformed phenotype in mutant p53/ras transformed cells (84). The oncogenic activity of mutants of p53 can be explained either by a dominant-negative effect of the overexpressed mutant over the endogenous wt protein (39)(23) or by a direct gain of function in p53-deficient cells(18).

HYPOTHESIS

The mechanism by which mutants of p53 acquire a gain of function is not well understood. There is evidence that transcriptional activation is involved. Unlike wt p53, however, transcriptional activation by mutants of p53 might not be dependent on sequence specific DNA binding since this activity has not yet been identified for these mutants (84). Most of the studies on the transactivation function of mutants of p53 has been performed by co-transfection of mutants of p53 and a reporter gene into cells lacking endogenous p53. In this manner, it has been shown that a series of oncogenic mutants can activate transcription of the PCNA promoter(15), the MDR-1 promoter (13) and the HIV-1LTR promoter (71). These data suggests that transactivation of normally inactive genes is a possible mechanism by which mutants of p53 can be involved in transformation.

If mutants of p53 are unable to bind to DNA in a sequence specific manner, how do these mutants activate transcription? Emerging evidence suggest the possibility that these mutants bind to a region of DNA that is different from the consensus sequence defined for wt p53 and that the binding is facilitated by the association with other proteins (30)(7). A p53 responsive element utilized by a mutant conformation of p53 has been identified in the HIV-LTR in a site near the Sp1 binding region. This element is required for TNF alpha induction of this promoter (30). TNF alpha induces the association of a wt p53 and Sp1 and Sp1 cooperates with this conformationally changed wt p53 in the transcriptional activation directed by this element (30)(31). Another cytokine, Granulocyte/Macrophage Colony-stimulating factor (GM-CSF), also induces the association between a conformationally changed wt p53 and Sp1 in human Erythroleukemia cell line (TF-1) and this heteroduplex formation mediates GM-CSF dependent proliferation of TF-1 cells (7). Interestingly, all the promoters activated by either mutants of p53 or the conformationally changed wt p53 share Sp1 binding sites in common.

These observation suggests that cytokines induce a mutant conformation of p53 that can associate with Sp1 and this heteroduplex might be involved in activating transcription of a subset of genes involve in cell proliferation. Mutation of the p53 gene might code for a protein that is constitutive in the conformation responsible for activating genes involved in cell proliferation. It is therefore of importance to define the DNA sequence from which

mutants of p53 might activate transcription. We intend to try to clarify the molecular mechanism by which oncogenic mutants of p53 work by defining the DNA sequence from which the mutant p53 His 273 activates transcription.

PURPOSE

To begin to define the DNA sequence from which mutants of p53 might activate transcription, *their vivo* footprinting of the HIV-LTR region transfected into the breast cancer cell line MDA-MB-468 and a normal breast cell line (MDA10A) will be compared. MDA MB 468 carries one deleted and one mutant p53 allele that codes for a protein in which an Arginine is substituted by a Histine at position 273(56)

The HIV-LTR region has been chosen for this study because, unlike other promoters, a minimal region required for transactivation by mutants of p53 has been reported (71)(31) and many of the proteins that bind to this region have been identified (28) for what antibodies are commercially available for their detection. DNase I footprinting analysis has been used to identify regions to which these factors bind(28). Additionally, a continuation of this study is to carry out the experiments described in an HIV-1 latently infected cell line which when transfected with mutant p53 His273 becomes actively infected (20).

The 273 mutant of p53 was chosen for this study for several reasons. It has been demonstrated in co-transfection experiments to activate transcription of a HIV-LTR reporter construct(71). This mutant has been classified as a contact mutant meaning that it does not bind to the wt p53 consensus sequences because it lacks a residue that is important for direct DNA contact(14). Understanding how this mutant gives cells a growth advantage might lead to the development of a targeted treatment method for tumors that contain p53 contact mutants

Intact mutant p53 His273 can bind non-specifically to the wt p53 consensus sequence at 20°C but, unlike wt p53, the thermolysin-resistant mutant fragment can not(5). This data suggest that mutant p53 His273 might bind specifically to different DNA sequences, that this binding might be temperature sensitive and therefore post-translationally modified and that sequences outside the core domain are involved in recognition of these sequences.

We have preliminary results indicating that HIV-LTR directed transcription significantly increases when MDA-MB-468 cells are shifted to 32°C (137 fold induction) as compared to 37°C (3.6 fold induction) (see Fig. 18a). This increase was inhibited if a ts p53Val 135 expressing plasmid was cotransfected along with the HIV-LTR reporter plasmid and the cells were shifted to 32°C (Fig 18 b), with the fold induction then being 2.2. Because TNF alpha induced activation of HIV-LTR directed transcription is mediated by the ability of this cytokine to induce heteroduplex formation between a conformational mutant of wt p53 and Sp1, it is possible that a temperature shift changes the conformation of 273 (the same way that TNF alpha induces a mutant conformation of wt p53) to a form that can bind to Sp1 and thus activate transcription from the HIT-LTR promoter.

Interestingly, although the HIV-LTR reporter construct demonstrates the highest transcriptional activity in MDA-MB-468 cells at 32°C when compared with an mdm2 reporter construct (2) and a construct with 16 p53 binding sites (RGCluc) (25), the

comparative activity from these promoter constructs in ZR 75-1 cells is dramatically different. The construct with 16 p53 binding sites (RGCluc) shows the highest transcriptional activity (143 fold induction) and mdm2 the lowest (52 fold induction, with the HIV-LTR falling in the middle (91 fold induction) (Fig. 18a), however when a ts p53Val135 construct is cotransfected along with the reporter plasmids the fold induction of mdm2 dramatically increases at 32°C (rising up to 681 fold induction) while being dramatically reduced for the HIV-LTR (dropping to 18 fold induction). These data suggest that the wild-type p53 in ZR 75-1 cells is not in a completely wild-type active form. Additionally they demonstrate that we can activate HIV-LTR driven transcription by the endogenous mutant p53 in the breast derived cell line MDA-MB-468 and can also repress HIV-LTR driven transcription by exogenous wild-type p53 in various breast derived cell lines.

STRATEGY for FUTURE HIV STUDIES

The procedures below will be carried out at 37 and 32 degrees Celsius. The results will be compared to those from a breast cancer cell line with no p53 (MDA-MB-157) as well as those from a normal breast cell line (MDA10A).

- *Confirm HIV-LTR transactivation by mutant p53 His 273 by introducing a HIV-LTR/luciferase construct into MDA-MB-468 cells.
- * Perform Gel mobility shift assays using MDA-MB-468 cell extracts and a 242 bp fragment from the HIV-LTR region.
- * Carry out immunoprecipitation of p53 from 468 cells extracts using monoclonal antibodies specific for different forms of p53.
- *Use the Promega Grab system to determine proteins that bind to the HIV-LTR in a mutant p53 His 273 dependent manner
- *DNase 1 sensitivity of the HIV-LTR region by southern blot.
- **In vivo* and *in vitro* footprinting of the HIV-LTR region by ligation mediated PCR

Is the transactivation from HIV-LTR dependent on the ability mutant p53 273H to bind to this region? If so, is this binding specific?

This question will be answered by using Electrophoretic mobility shift assay (EMSA) with extracts of four different breast cell lines that have different p53 status. A fragment from the HIV-LTR containing the putative p53 responsive element(31) will be used. This fragment is 242 base pairs and will be obtained by digesting the HIV-LTR region with HindIII/AvaI. The fragment will be labeled by Random Priming. Extracts will be made from MDA-MB-468 cells grown at 37 and 32 degrees Celsius. The presence of p53 will be tested by the addition of different p53 monoclonal antibodies. Additionally, the presence of other factors that bind to this region will also be tested by addition of specific antibodies. The dependence of p53 for binding of these other factors will be determined by using extracts from MDA-MB-157 cells, a p53 negative cell line, ZR75.1, a wt p53 cell line and the normal breast cell line MDA10A. The dependence of other factors for binding of this mutant p53 will be tested by using purified proteins. Since Sp1 is suspected to be involved, purified Sp1 will be used either alone or in combination with this mutant p53. The specificity of the binding will be determined by competition experiments with unlabeled fragment. The

inability of this mutant to bind to the sites bound by wt p53 will be confirmed by performing EMSA comparing the various extracts with an oligo containing the mdm2 gene wt 53 binding site.

Is the binding of mutant p53 His 273 to the HIV-LTR facilitated by the association with other proteins?.

This question will be approached by using co-immunoprecipitation and the Promega Grab technique. These approaches will allowed us to study the alternated pathway by which mutants of p53 might activate transcription. It is possible this mutant associates with other DNA binding proteins and through its activation domain activates transcription. A GAL4/mutant p53 His 273 fusion protein has been shown to activated transcription from a GAL4 responsive element (63). Additionally, the association of a mutant conformation of p53 with Sp1 has been shown to activate transcription from the HIV-LTR (30). The HIV-LTR has three Sp1 binding sites (28). Interestingly, when these Sp1 binding sites were identified, it was suspected that other cellular factors could bind to and regulate HIV-1 gene expression via this sites.

Immunoprecipitation from extracts of the three cell line will be carry out with monoclonal antibodies that recognized different forms of p53. It has been observed that two novel size proteins that are not yet identified co-immunoprecipitated with mutants of p53(11). The immunoprecipitate will be separated by SDS-PAGE and Western Blotted with an antibody against Sp1. Immunoprecipitation will also be carry out with an Sp1 antibody and Western Blotted with a p53 antibody.

The GRAB system uses the binding of the Lac Repressor to the Lac Operator as the basis for isolating factors that bind to sequences placed adjacent to the Lac operator (48). Briefly, the HIV-LTR fragment will be cloned adjacent to the Lac Operator in the plasmid pGEM-3f(-). This fragment/operator oligo will be incubated with a Lac Repressor/Beta-galactosidase fusion protein. This complex will be immobilized by running through a solid matrix containing an antibody against Beta-galactosidase. Cell extracts from the three cell lines with different p53 status will be pass over the column. The fragment containing bound proteins will be release by adding Isopropyl-B-D thiogalactopyranoside (IPTG) which causes the repressor to release binding to Operator. The isolated factors will be analyzed, both by size, using SDS-PAGE, and by antibody screening, using Western Blotting. The total proteins isolated will also be examined by biotin labeling of isolated factors follow with detection. This task of identifying isolated factors will be facilitated by the availability of antibodies for many of the factors that bind to this region. This makes the task one of determining the dependence on the mutant p53 for binding of the factors and not one of identifying new ones.

If mutant p53 His 273 activates transcription by binding to DNA, what is the DNA sequence to which it binds? Is the activation through binding to Sp1, does the association of this mutant p53 with Sp1 alters the way Sp1 binds to DNA?

This question will be approached by DNase I *in vitro* and *in vivo* footprinting of the binding region by ligation mediated PCR. This technique will be used because it allows for single-copy genomic cleavage by DNase I to be detected at the nucleotide level of resolution (55). This will allow for the DNA sequence protected in a mutant p53-dependent manner to be determined. This technique has been used *in vitro* (80) and *in vivo* (34)(64)(53) to identify the sequence in the HIV-LTR protected by other factors such as Sp1 (34).

Conclusions:

During months 12-24 we have succeeded in addressing many of the questions set forth in the grant proposal. We are selecting for the stable breast cancer lines expressing the temperature sensitive p53 val135 mutant and have selected such a line in the mouse embryo fibroblasts cells.

The mdm2 gene has two promoters, the first (P1) is constitutively active while the second (P2) can be activated by wt p53 via a sequence-specific interaction. Two putative p53 response elements (p53-RE's) that share limited degrees of homology with the defined consensus sequence have been identified in the P2 region. We have investigated the *in vivo* protein interactions that occur within this region. To facilitate our study all experiments have been done in both a cell line lacking p53 and in a derivative cell line containing temperature sensitive p53val135. Comparing the DNA-protein interactions and chromatin structure of this region in the presence and absence of p53 by monitoring DNaseI sensitivity with Southern blot analysis, and utilizing *in vivo* footprinting, we have identified when p53 induced differential sensitivity exists and the sequences protected by protein in the nuclear chromatin. At 39°C, when this ts p53 mutant is predominantly localized to the cytoplasm, we observe no major difference. However after the cells have been shifted to 32°C for 4 hours and the p53 is predominantly localized to the nucleus, the DNaseI sensitivity and *in vivo* footprinting patterns for the two cell lines clearly differ. Each putative p53 response element in the ts p53 containing cell line is protected over its downstream half while the same sequence is unprotected in the cell line without p53. Additionally, upon binding of p53 to the p53 RE's, noticeable changes in protection occur over the adjacent 3' region which contains the TATA box. Surprisingly, when the cells are left at 32°C for 24 hours, a decrease in protection of the p53-RE's is noted with a concomitant extended area of protection resulting over the adjacent 3' region. Using transient transfection experiments with a construct containing the P2 region we have observed that greater activation results after 24 hours at 32°C than after 8 hours, however the increase is only 1.5 fold. A monoclonal antibody specific for p53 (PAb 421) is able to induce a p53 specific gel shift when added to nuclear extract derived from the ts p53 cell line shifted to 32°C for either 2 or 4 hours, but not when added to extract from cells placed

at 39°C. Interestingly, this shift decreases in level and size when the cells are left at 32°C for 24 hours. Taken together these data suggest that p53 can successfully recruit the transcription machinery directly after binding to the p53 RE's, and after a period of time p53 changes its interaction with the binding elements while influencing a change in the proteins interacting with the downstream DNA.

Additionally, in the breast cancer cell lines ZR75-1 (wtp53 +/-), MDA-MB-468 (273 Arg to His) and MDA-MB-157 (null for p53 by deletion), we have compared the transcription activity from three reporter constructs (one with 16 p53 RE's, one with the mdm2 P2 promoter and one containing the HIV-LTR). In ZR75-1 the multiple p53 RE construct gives the highest luciferase activity, suggesting that some wild-type p53 activity exists in these cells. In MDA-MB-468 the HIV-LTR construct gives the highest luciferase activity while in MDA-MB-157 the levels of all three constructs are approximately the same. This suggests that mutant p53 His273 in breast cancer cells is able to activate transcription of the HIV-LTR in a similar manner to what has been published for cotransfection of His273 p53 with the HIV-LTR construct. Although wild-type p53 activity exists in ZR75-1 we have been unable to "activate" this p53 activity with chemotherapeutic DNA damaging drugs. Experiments are in progress to *in vivo* footprint the mdm2 P2 promoter, the HIV-LTR promoter and the gadd45 promoter in these three breast cancer cell lines and to compare them to footprints in normal breast cell lines.

We have succeeded in isolating pure cell cycle fractions from exponentially growing hematopoietic cell lines and have encouraging preliminary data from the nuclear cell extracts. These data will be compared to those for the breast cancer cell lines.

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Table 1

Days after shift Cell cycle disribtion % Days after shift Cell cycle disribtion %

	% G1	% S	% G2/M		% G1	% S	% G2/M
10(1) 37°C	37.2	49.2	13.7	3(4) 37°C	21.4	57.5	21.2
10(1) 32°C Day 1	59.5	33.8	7.4	3(4) 32°C Day 1	31.9	12.8	55.3
10(1) 32°C Day 2	65.4	25.2	9.4	3(4) 32°C Day 2	38.3	17.0	52.6
10(1) 32°C Day 3	72.2	16.6	11.2	3(4) 32°C Day 3	23.7	23.6	52.7
10(1) 32°C Day 4	32.0	61.8	7.0	3(4) 32°C Day 4	30.9	13.5	55.7
10(1) 32°C Day 7	44.6	42.0	13.3	3(4) 32°C Day 7	21.3	18.2	60.5

Fig. 1a

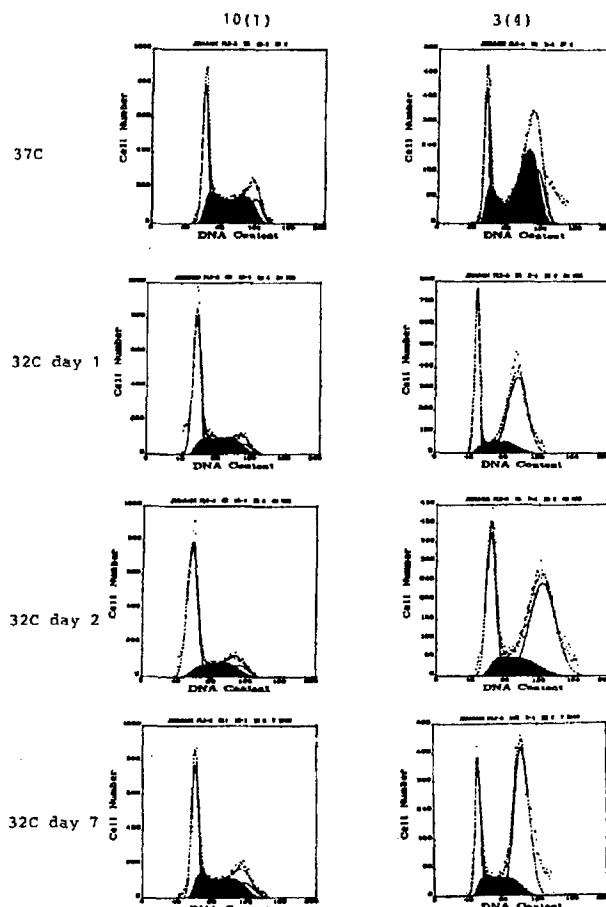


Fig. 1b

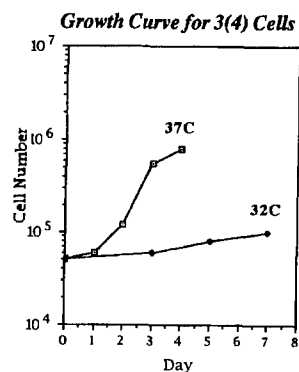


Figure 1 Growth analysis of 10-1 and 3-4 cell lines.

- a) FACS analysis showing cell cycle distribution of 10-1 and 3-4 cells at permissive temperature(37°C) and restrictive temperature(32°C) at indicated time.
- b) Growth curve of 3-4 cells indicate the growth arrest at restrictive temperature.

Fig. 2a

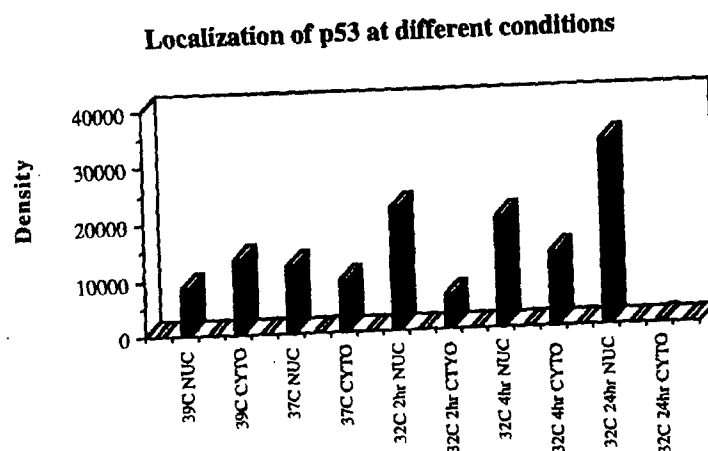


Fig. 2b



Fig. 2c

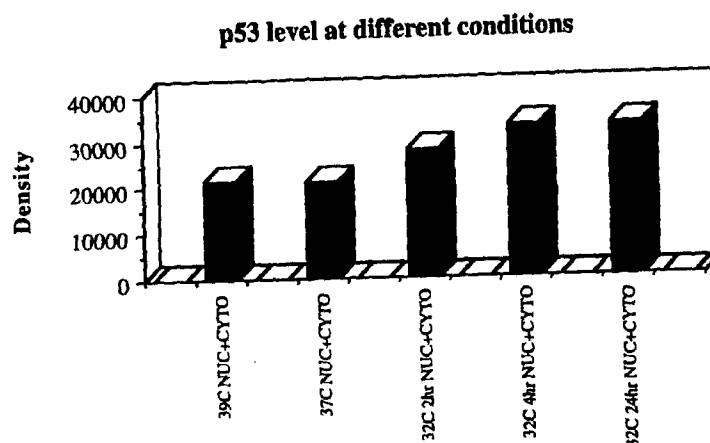


Figure 2 Subcellular levels of p53 in 3-4 cells at 39°C, 37°C and 32°C.

- a) Quantitation analyze of nuclear and cytoplasmic distribution as determined by densitometer analysis and image Quant software(molecular Dynamics).
- b) Western blot showing the protein localization at 39°C, 37°C and 32°C. Proteins were immunoprecipitated using PAb421 crosslinked to protein A sepharose.
- c) Comparison of the total amount p53(nuclear plus cytoplasmic) at the various temperatures and time points.

Fig. 3a

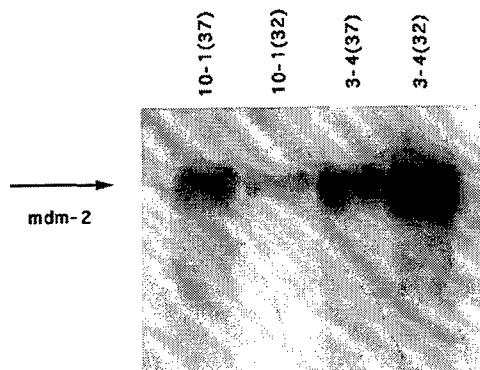


Fig. 3b

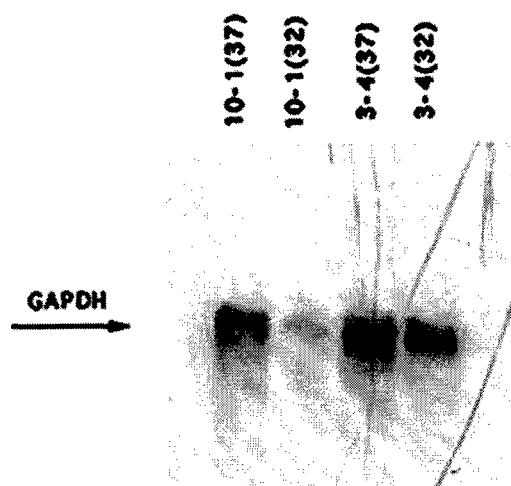


Figure 3 Northern blot showing mRNA level of 10-1 and 3-4 cells at 37°C and 32°C for 4 hours.

1.8ug of poly-A mRNA was separated by electrophoresis on 1% formaldehyde agarose gel. The mRNA was transferred to positively charged nylon membrane and probed with a) an mdm-2 genomic DNA probe; b) The membrane was then stripped and reprobed with a GAPDH cDNA probe.

Fig. 4

Immunoprecipitated p53 levels in breast cancer cell lines.

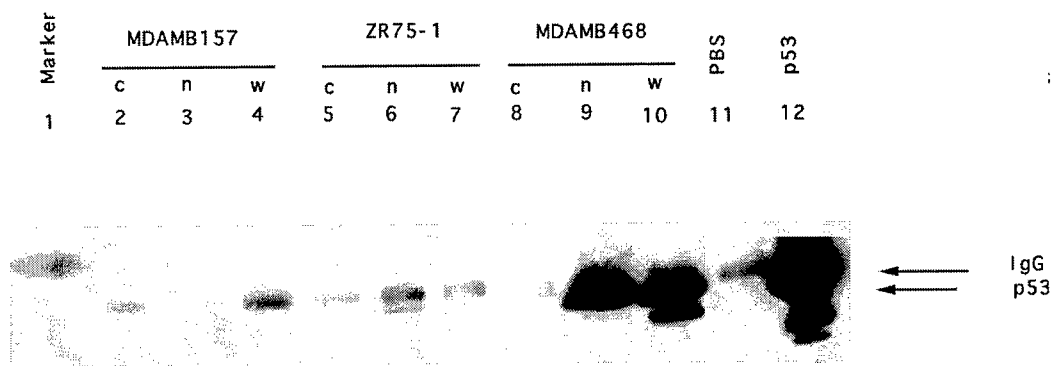


Figure 4: Levels of p53 in breast cancer cell lines.

Immunoprecipitation was carried out as described in methods. Lane 1 corresponds to prestained molecular weight marker, lanes 2-4 correspond to MDA-MB157 cytosolic, nuclear and whole cell extract respectively; lanes 5-7 contain ZR-75.1 cytosolic, nuclear and whole cell extract respectively; lanes 8-10 contain MDA-MB 486 cytosolic, nuclear and whole cell extract respectively; lane 11 contains beads rocked with PBS and lane 12 has purified p53 standard. IgG (a background protein) is also indicated.

Figure 5

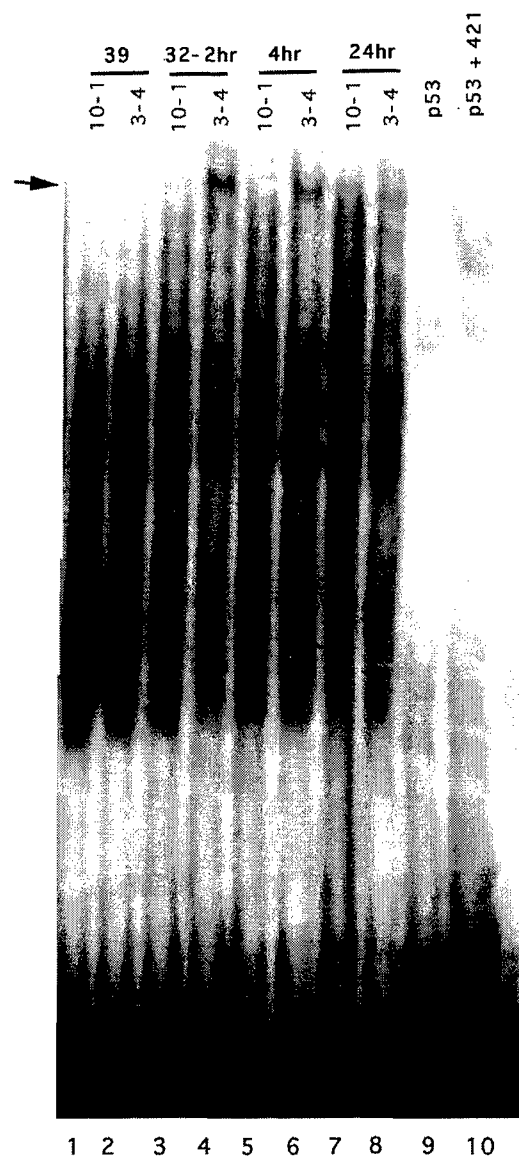


Figure 5: EMSA of an mdm2 oligonucleotide varies for 3-4 nuclear extracts prepared after different times at 32°C. Reactions were carried out as described in the methods with nuclear extracts from 10-1 and 3-4 cells (plus PAb421) at 39°C (lanes 1 and 2), at 32°C for 2 hours (lanes 3 and 4), at 32°C for 4 hours (lanes 5 and 6) and at 32°C for 24 hours (lanes 7 and 8). Reactions in lanes 9 and 10 contain purified p53 with and without PAb421 respectively.

Fig. 6a

mdm-2-probe

	10(1) 39C						3(4) 39C					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16

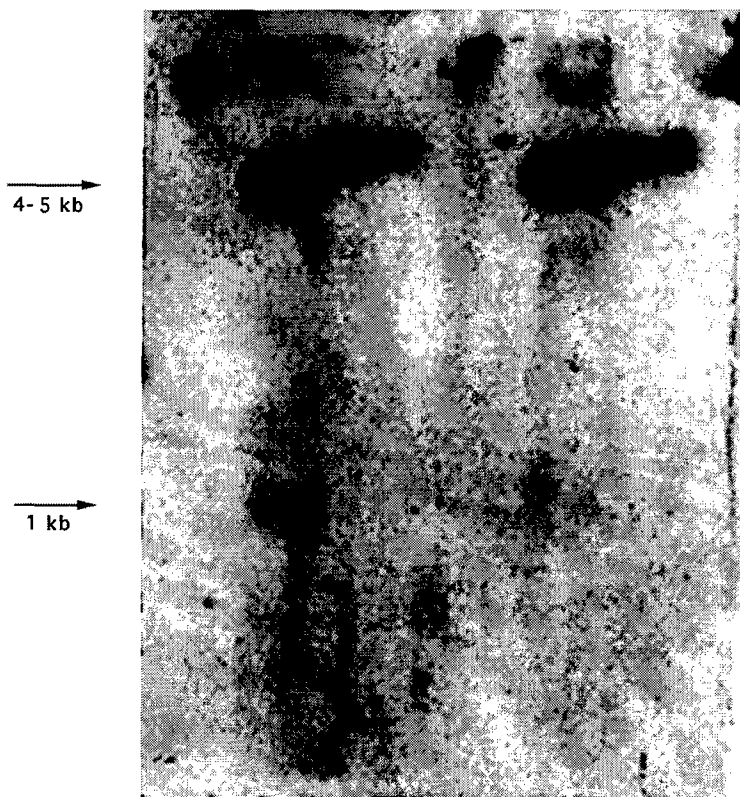


Fig. 6b

GAPDH-probe

	10(1) 39C						3(4) 39C					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16

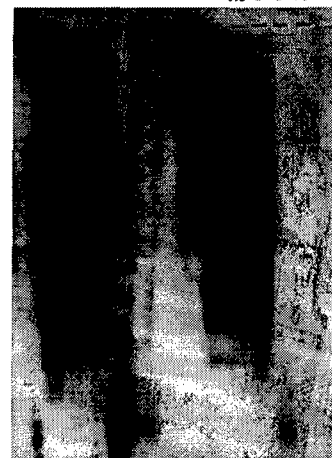


Figure 6 Southern blot showing different DNase I sensitivity at mdm-2 promoter region (P2) in 10-1 and 3-4 cells.

15ug DNA samples labeled as indicated were electrophoresed on a 1% agarose gel. The DNA was transferred to nylon membrane and probed first with mdm-2 genomic DNA probe(Figs. a, c, e). The membrane was subsequent stripped and reprobed with GAPDH cDNA probe(Figs. b, d, f).

Fig. 6c

mdm-2-probe

	10(1) 32C 4hrs						3(4) 32C 4hrs					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16

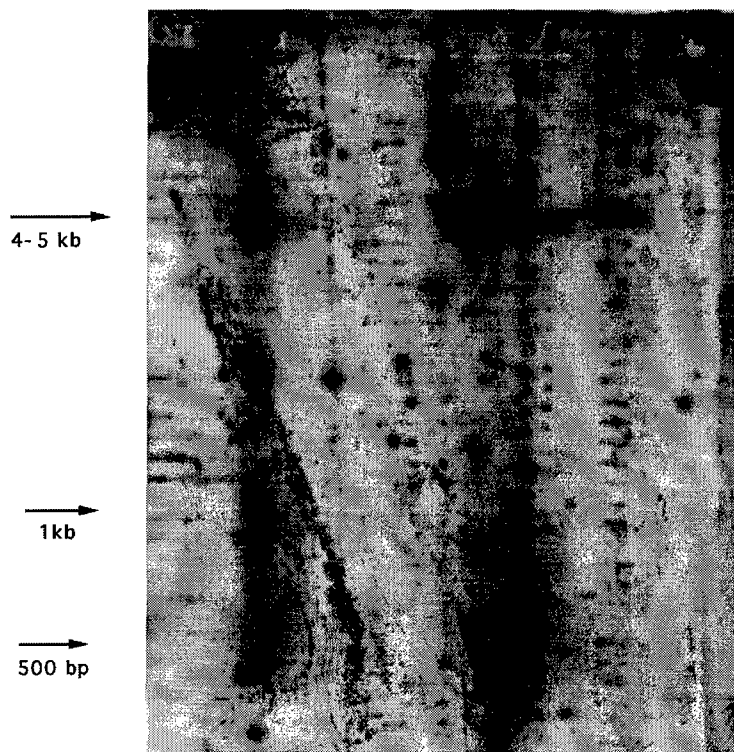


Fig. 6d

GAPDH-probe

	10(1) 32C 4hr						3(4) 32C 4hr					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16



Fig. 6e

mdm-2-probe

	10(1) 32C 24 hr						3(4) 32C 24hr					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16



Fig. 6f

GAPDH-probe

	10(1) 32C 24hr						3(4) 32C 24hr					
Nsi I	-	+	+	+	+	+	-	+	+	+	+	+
DNase I (ug)	-	-	0.5	2	8	16	-	-	0.5	2	8	16



Sequence Range: 1 to 585

Fig. 7a

```

50
--gggcccgc tccgggtcgc gctggctcgt tgctgggtcc aggaggtgac aggtgcctgg tcccggactc
--cccggggc agggcccagca cgcaccgagca acgaccagcgc tctccactg tccacggacc agggcctgag

100
gccgggatgc ggcttcgggg acgggtggga ctggtctggg ccgagttgac tcagctcttc ctgtggggct
cggccctacg ccgaaggccc tgcccaccct gaccagaccg ggtcaactg agtcgagaag gacaccccga

150
ggtaagttg ggacacgtcc ggcgtcggtc gtcggaggag ctaagtccctg acatgtctcc agctgggggt
ccagttcaac cctgtgcagg ccgcagccga cagctctctc gattcaggac tgtacagagg tcgaccccaa
P53 RE1 P53 RE2

250
atttaaacgc tccccggtt ccgcagccgt ctgctggggc agcgggagac cgaccggaca cccctggggg
taaatctgag acggggcaaa ggcgctggga gacgaccgcg tcgcccctcg gctggcctgt ggggaccccc

300
accctctcgg atcaccgcgc ttctcctcgc ggctccagg taaggagacg ctgcgcgacg tcgtttttgca
tgaggagacc tagtggcgcg aagaggacgc ccggaggtcc attcctctgc gacggcctgc agcaaaacgt

400
tttgagagct attgccgaaa gacgttttct gtctccttcg taaatgcagc tatctatttg tcccttttcg
aaactctcga taacggcttt ctgcaaaaga cagaggaagc atttacgtac atagataaac agggaaaagc

450
tagatgtttt ataatttcaa gtttttatcg tgtgtgtgtg tgtttttttt ctactttagt gccaatgtgc
atctacaaaa tattaaagtt caaaaatagc acacacaaca acaaaaaaaa gatgaacatc cggttacacg

500
aataccaaca tgtctgtgtc tacgaggggt gtcgcaagca gctcacagat tccagcttcg gaacaagaga
ttatggttgt acagacacag atggctccca cgacgttcgt ggagtgctca aggtcgaagc ctgtttctct
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gagaccaacc ataaggtgg agctc
oligo*1 oligo*2 oligo*3

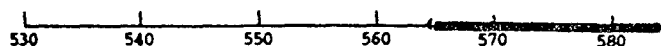
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Fig. 7b

The design of three oligonucleotides for Ligation-mediated PCR *in vivo* footprinting:

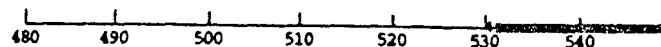
Oligo#1

584-565 5'- TCGAGGTAGAAATACCAACC -3'
20 nt primer on minus strand
pct G+C: 45.0 Tm: 52.3



Oligo#2

550-531 5'- CGAAGCTGGAATCTGTGAGG -3'
20 nt primer on minus strand
pct G+C: 55.0 Tm: 55.3



Oligo#3

543-520 5'- GGAATCTGTGAGGTGCTTCAGCA -3'
24 nt primer on minus strand
pct G+C: 54.2 Tm: 60.8

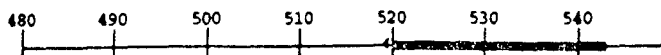


Fig. 8a

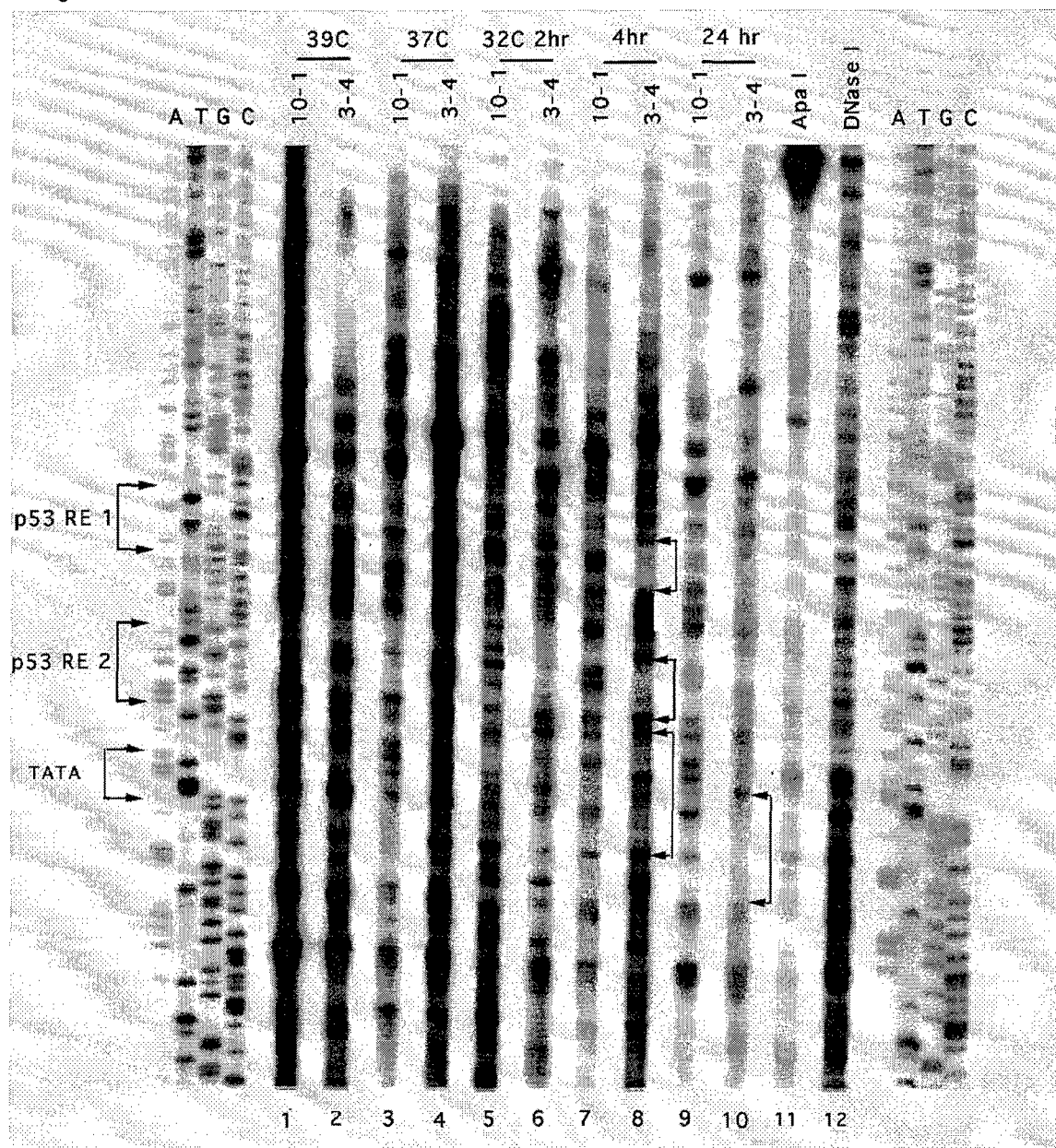


Figure 8a Ligation-mediated PCR *in vivo* footprinting showing the p53 binding site on mdm-2 promoter (P2) *in vivo*.

The published p53 responsive elements and TATA box are identified by sequencing the genomic mdm-2 plasmid DNA (first 4 lanes: A, T, G, C). The samples electrophoresed in each lane corresponding to the *in vivo* footprinting reactions of 10-1 and 3-4 cells at different temperatures are as indicated above.

Fig. 8b

The published p53 responsive element 1:
p53 RE-1(140-160)

*	*	*
5'-GGTCAAGTTG	GG/ACACGTCC/-3'	
3'-CCAGTTCAAC	CC/TGTGCAGG/-5'	
*	*	*
3'-Py Py Pu GTTC Pu Pu Py	Py Py Py GTGC Pu Pu Pu-5'	

The protection region from our data (151-164)

5'-/ACACGTCC/GGCGT-3'
3'-/TGTGCAGG/CCGCA-5'
*
3'-GTGC Pu Pu Pu-5'

The published p53 responsive element 2:
p53 RE-2 (178-198)

* *	*
5'-AGCTAA/GTCC	TGACATGTCT/-3'
3'-TCGATT/CAGG	ACTGTACAGA/-5'
* *	*
3'-Py Py Pu ATTC Pu Pu Pu	Pu Py PyGTAC Pu Pu Pu-5'

The protection region from our data (184-198)

5'-/GTCC	TGACATGTCT/-3'
3'-/CAGG	ACTGTACAGA/-5'
3'-GTAC Pu Pu Pu -5'	

*: mismatch
/: common sequence

Figure 8b Comparison of the published putative binding sequence and the sequence attended by intranuclear footprinting of the mdm-2 P2 promoter region.

Sequence Range: 1 to 500

Fig. 9a

```

50
ggctgcgggc cctgcggcg cgggaggtcc ggaatgatgc aggtgcctgt cgggtcacta gtgtgaacgc
cgcacgcccg gggacgcccg gccctccagg cctactagcg tccacggaca gccacgtgat cacacttgcg

100
tgcgcgtagt ctgggcggga ttgggcccgt tcagtgggca ggttgactca gcttttcttc ttgagctggt
acgcgcacat gacccgccct aaccgggcca agtcacccgt ccaactgagt cgaaaaggag aactcgacca

150
caagttcaga cacgttcgga aactgcagta aaaggagtta agtccctgact tgcctccagc tggggctatt
gttcaagtct gtgcaaggct ttgacgtcat tttctcaat tcaggactga acagaggtcg accccgataa
p53RE1 p53RE2

250
taaaccatgc attttcccag ctgtgttcag tggcgattgg agggtagacc tgtgggcacg gacgcacgcc
atttggtagc taaaagggtc gacacaagtc accgctaacc tccatctgg acacccgtgc ctgcgtgcgg

300
actttttctc tgcctgatca ggtaaggacc gacttgcctg tagcttagt ttttaactgtt gtttatgttc
tgaaaaagag acgactaggt ccattcgtgg ctgaacgaac atcgaaatca aaattgacaa caaatacaag
oligo#3 oligo#2

400
tttatatatg atgtattttc cacagatgtt tcatgatctc cagttttcat cgtgtctttt ttttcttgt
aaatatatac tacataaaag gtgtctacaa agtactaaag gtcaaaagta gcacagaaaa aaaaggaaca

450
aggcaaatgt gcaataccaa catgtctgta cctactgatg gtgctgtaac cacctcacag attccagctt
tccgtttaca cgttatggtt gtacagatat ggaatgactac cagcacattg gtggagtgtc taaggctcga
oligo#1

500
cggaacaaga
gccttgttct

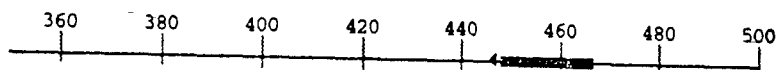
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Fig. 9b

The design of three oligonucleotides for Ligation-mediated PCR *in vivo* footprinting in human breast cancer cell lines:

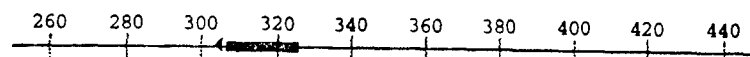
Oligo#1

467-448 5'- ACAGCACCATCAGTAGGTAC -3'
20 nt primer on minus strand
pct G+C: 50.0 Tm: 51.5



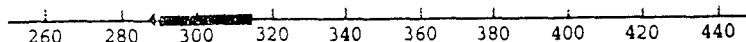
Oligo#2

326-307 5'- AAGCTACAAGCAAGTCGGTG -3'
20 nt primer on minus strand
pct G+C: 50.0 Tm: 57.5



Oligo#3

315-290 5'- AAGTCGGTGCCTTACCTGGATCAGCAG -3'
26 nt primer on minus strand
pct G+C: 53.8 Tm: 60.3





3781 gactttcagc cgagatgtgc tagtttcac accaggattt tctgtggtac a**GAACATGTC**
3841 **TAAGCATGCTG**gggactgccagcagcggaagagatccctgtgagtcagcagtcagcccag
3901 ctactcccta cctacatctg *cactgcctcc cgtgactaat* **tcctttagca** gggcagaita
3961 gataaagcca aatgaattcc tggctcacc ctcattaagg agtcagcttc attctctgcc
4021 agtcagagct aaaaatagaa attgtgtagg agacaaacct tgtaattcc ctagaaatac
4081 attaagagga tagagtggaa ttttttct ctgcaatctt gcatttttt aatggctctt
4141 tttttttc ctgataaaaa cctttggtag gtagggaagt tatgttttca ggggtaaatg
4201 tgctactttt gtcttctaaa tttgtcttt tttgactgg *tctagtcaag tgacagoccg*
4261 attattttgc tactccttaa aagtactatt ctgtctcttg gaqtatgqtt gatqgcaatt

Oligonucleotides designed for gadd45 analysis by LM-PCR

SEQUENCE: 5' gct gtc act tga cta gac ca 3'
Length 20bp (4257-4238)
COMPOSITION: %GC=52.6 Tm=52.2°C
No stable secondary structures
Homodimer Tm=8.3°C

SEQUENCE: 5' atc tgc cct gct aaa gga att ag 3'
Length 23bp (3958-3936)
COMPOSITION: %GC=43.5 Tm=60.1°C
Stem loop structure Tm=31°C
Homodimer not stable

SEQUENCE: 5' **aag gaa tta gtc** acg gga ggc agt gc 3'
Length 26bp (3945-3920)
COMPOSITION: %GC=53.8 Tm=70.1°C
No stable secondary structure
Homodimer Tm=0.7°C

1. overlapping regions of Oligo #2 and Oligo #3 are highlighted
2. all oligos run through GeneBank and matched only to human gadd45 gene

Figure 11: Levels of p53 in a Drug Treated ZR 75-1

Quantitative analysis of Results from Western Blots. The X axis corresponds to the different samples shown in the Western Blot and the Y axis is given in pixel units (proportional to the size and intensity of the bands in the Western Blot detection by ECL reagent), as determined by laser densitometer analysis and Image Quant software (Molecular Dynamics). **a) Treatment with DNA damaging agents.** ZR 75-1 cells were grown in RPMI 1640 medium with 10% fetal calf serum at 37°C with 5% CO₂ until they reached 70% confluence; at this point the cells were incubated with medium containing DNA damaging drugs as indicated. After 2, 4, 8 and 28 hours the cells were harvested and nuclear extract was prepared and samples were electrophoresed on a 10% SDS-PAGE and transferred for Western blotting with a mixture of p53 specific antibodies PAb1801, PAb421 and PAb240. All lanes were normalized for 60ug of total protein. Lanes 3, 7, 11 and 15 contain extract from cells treated with etoposide 10 ng/ml for the time periods as indicated. Lanes 4, 8, 12, and 16 contain extract from cells treated with etoposide 5 ng/ml for the time periods as indicated. Lanes 5, 9, 13 and 17 contain extract from cells treated with actinomycin D 1uM for the time periods as indicated. Lanes 6, 10, 14 and 18 contain extract from cells treated with camptothecin 10 uM for the periods of time as indicated. Lane 1 corresponds to the molecular weight marker and lane 2 is the control for cells without drug treatment. **b) Treatment with 2-LLL-CHO.** Cells were grown until 70% confluence and were then treated with 2-LLL-CHO at the concentrations and for the times as indicated. Nuclear extract was prepared and 500ug of total protein was immunoprecipitated with p53 specific antibody PAb421 cross-linked to protein A-sepharose beads. Immunoprecipitates were resolved on a 10% SDS-PAGE Western blot in the order as follows: 2.5 uM treatment for 1hr., 2hrs., 4hrs., 7hrs., and 24hrs (lanes 3-7 respectively) and 5.0 uM treatment for 1hr., 2hrs., 4hrs., 7hrs., and 24hrs (lanes 8-12 respectively). Lane 1 corresponds to the molecular weight marker, lane 2 to untreated cell extract, lane 13 to no cell extract and lane 14 to immunopurified p53. The 55kD IgG background band from immunoprecipitation is indicated.

Figure 11a

p53 protein level in LLL-CHO treated ZR75.1 cells

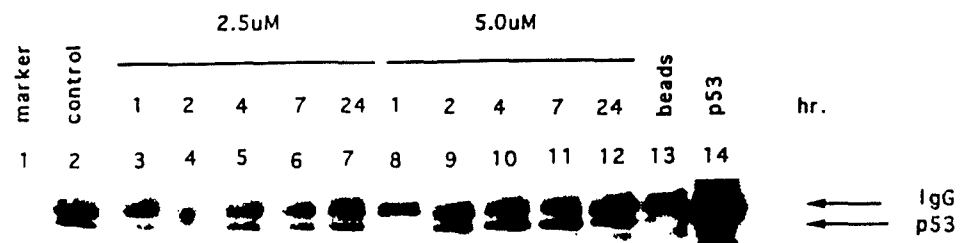
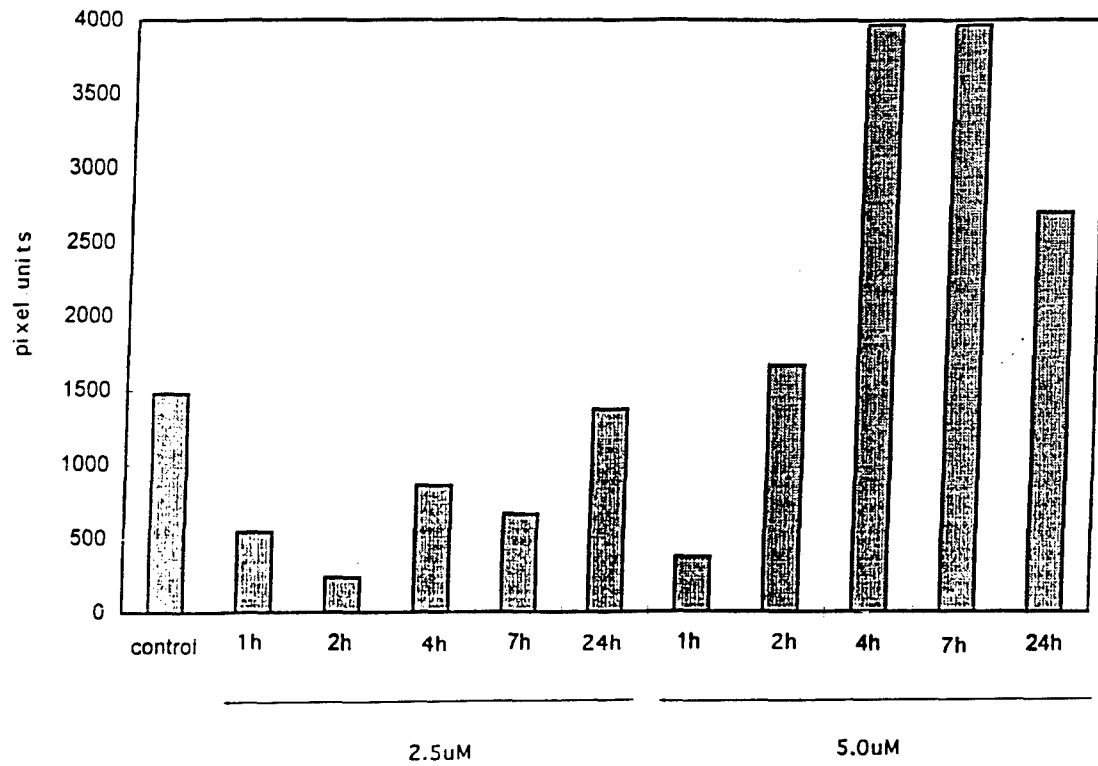


Figure 11b

p53 protein level in drug treated ZR75.1 cells

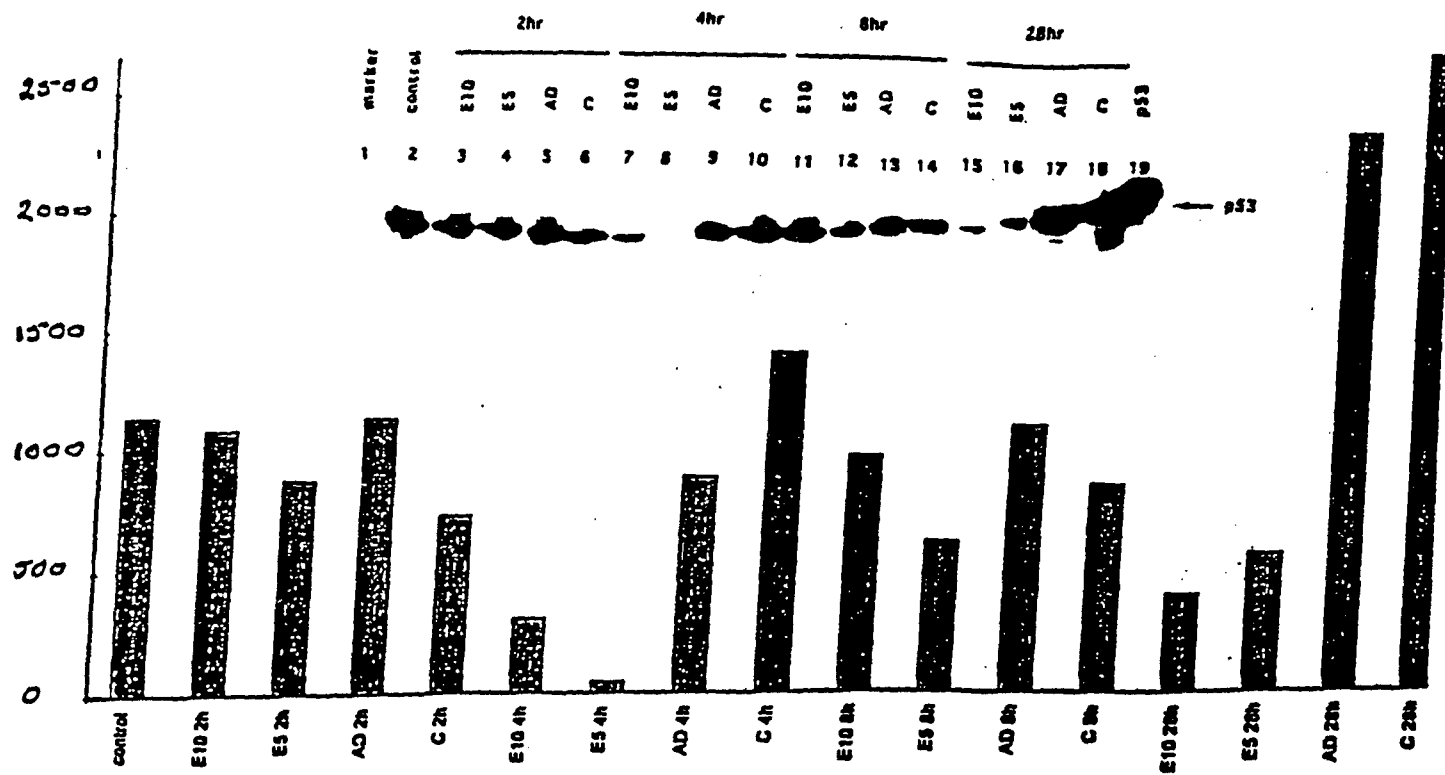


Figure 12a

ZR75-1 cells treated with Actinomycin D. EMSA assay with RGC

Mix	p53			His273			c			2			4			8			Hr.
	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	421
																			1801

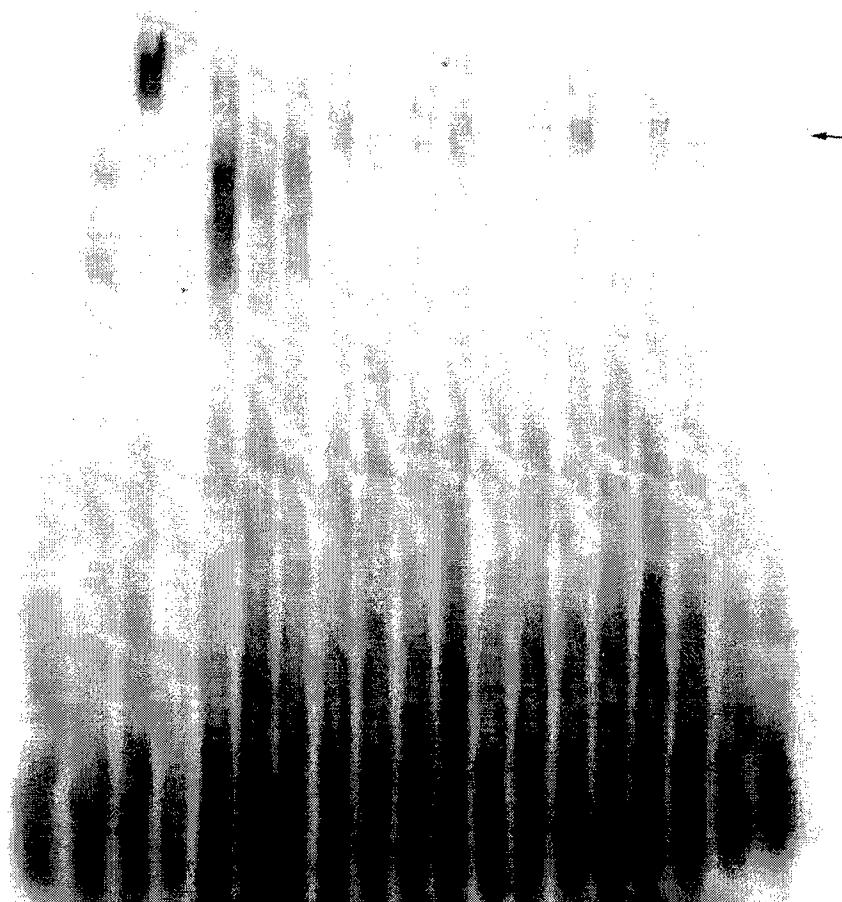


Figure 12a: Electrophoretic Mobility Shift Assay for Actinomycin D treated ZR75.1 cells. Cells were grown in RPMI 1640 medium, 10% FBS and 5% CO₂ at 37°C until they reached 70% confluence, then they were treated with Actinomycin D and nuclear extracts were made at the indicated times. The reactions were normalized for 2 ug of total protein per reaction, 150 fmol of ³²P labeled probe (RGC) per reaction.

Lane 1 - no protein; lane 2 - purified p53; lane 3 - purified p53+PAb1801; lane 4 - purified p53+PAb421; lane 5 - His273 mutant control; lane 6 - His273 control+PAb421; lane 7 - His273 control+PAb1801; lane 8 - control; lane 9 - control+PAb421; lane 10 - control+PAb1801; lane 11 - 2hours; lane 12 - 2hours+PAb421; lane 13 - 2hours+PAb1801; lane 14 - 4hours; lane 15 - 4hours+PAb421; lane 16 - 4hours+PAb1801; lane 17 - 8 hours; lane 18 - 8hours+PAb421; lane 19 - 8 hours+PAb1801;

ZR75-1 cell treated with Actinomycin D for 24 hrs
EMSA with RGC.

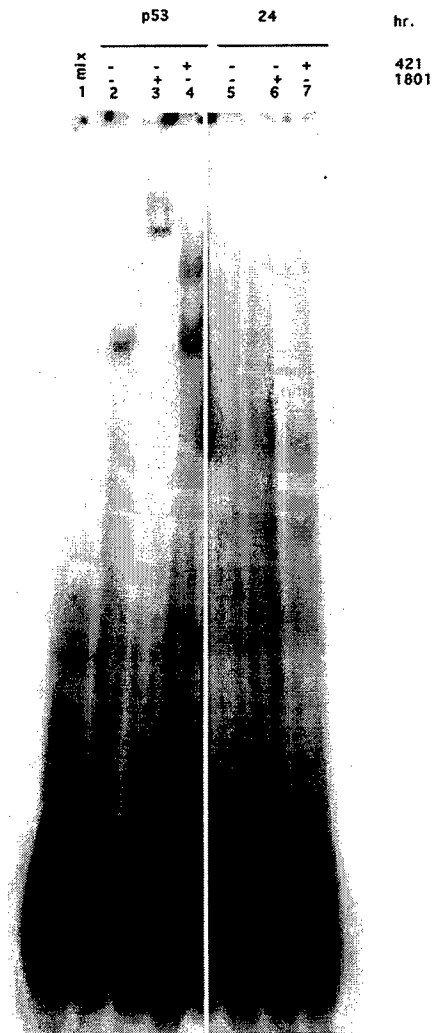


Figure 12b: Electrophoretic Mobility Shift Assay for Actinomycin D treated ZR75.1 cells. Cells were grown in RPMI 1640 medium, 10% FBS and 5% CO₂ at 37°C until they reached 70% confluence, then they were treated with Actinomycin D and nuclear extracts were made at the indicated times. The reactions were normalized for 2 ug of total protein per reaction, 150 fmol of ³²P labeled probe (RGC) per reaction.
Lane 1 - no protein; lane 2 - purified p53; lane 3 - purified p53+PAb1801; lane 4 - purified p53+PAb421; lane 5 - 28 hours treatment cell extract; lane 6 - 28 hours+PAb1801; lane 7 - 28 hours+PAb421.

Figure 13a

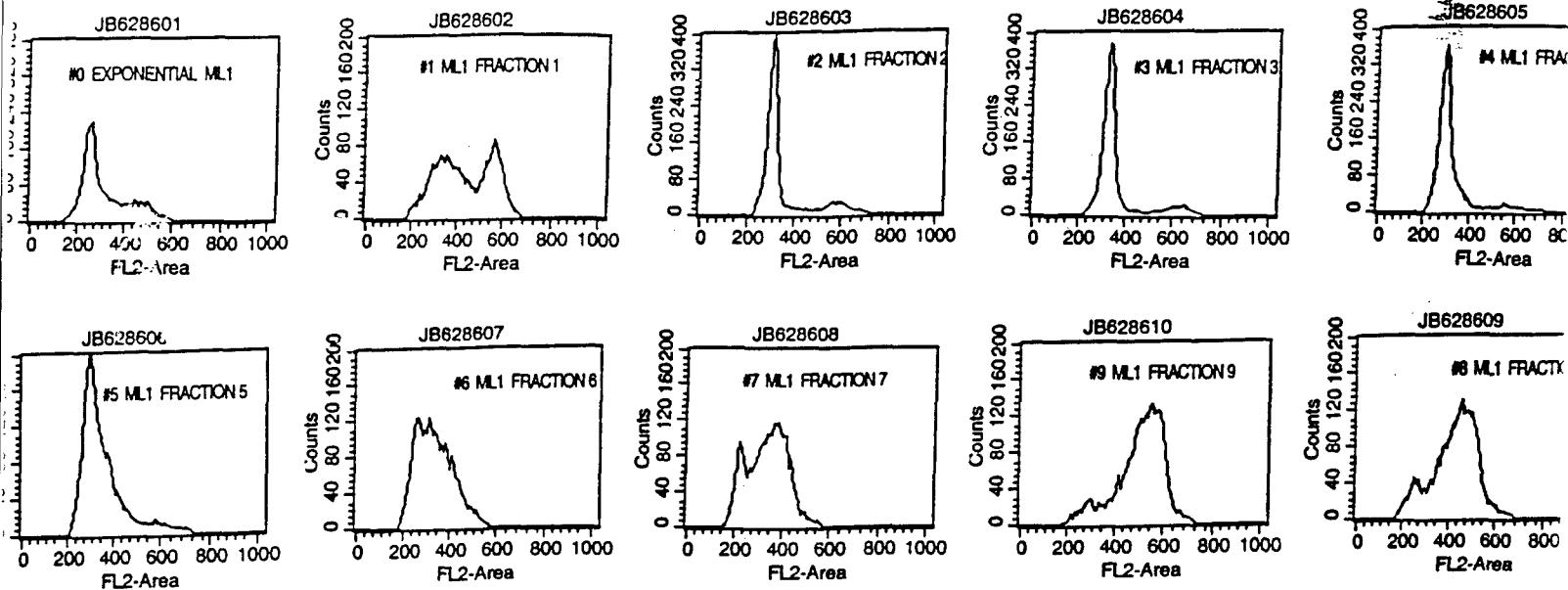


Figure 13b

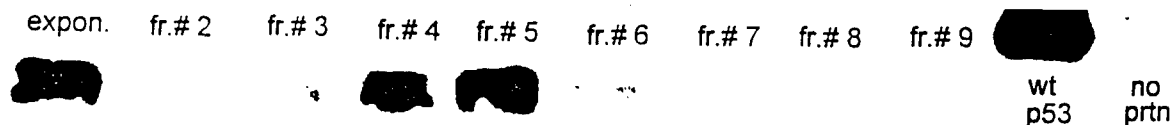


Figure 13c

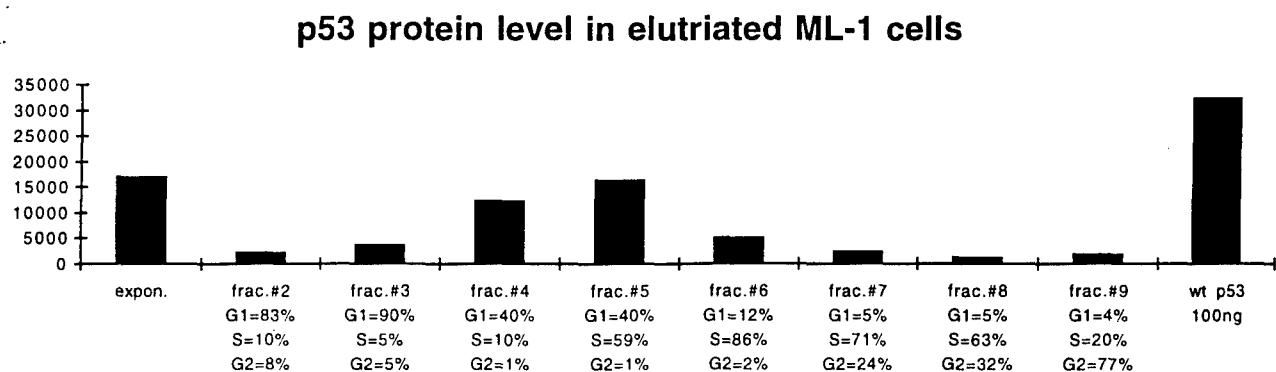


Figure 13: p53 protein levels in centrifugal elutriated ML-1 cell fractions.

Four liters of ML-1 cells at 4×10^5 cells/ml were elutriated using a Beckman elutriation rotor system.

A) The profile of the elutriated fractions was determined by FACS analysis. Nuclear extracts were prepared and immunoprecipitated as described in the methods. **B)** The p53 present in the immunoprecipitate was resolved by 10% SDS-PAGE and visualized by Western blot with a mixture of p53 specific antibodies PAb421, PAb1801 and PAb240 followed by detection with ECL reagent. **C)** The relative levels of p53 are presented as a histogram of pixel values determined by laser densitometer analysis and quantitation by Image QuANT software (Version 4.1).

Figure 14a: p-53 protein level in drug treated ML-1, HL-60 and MANCA cells

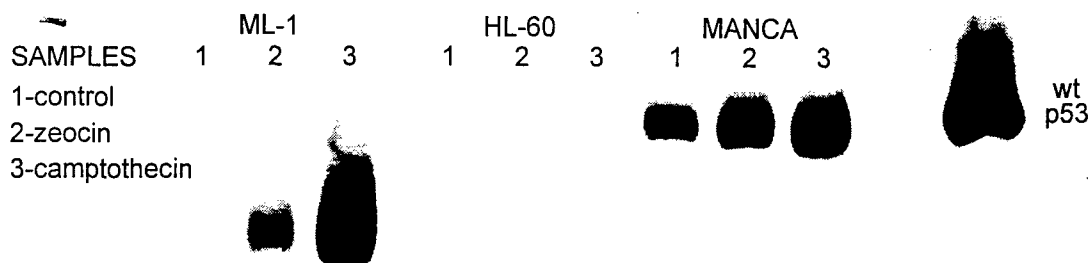


Figure 14b: Drug treatment of ML-1, HL-60 and MANCA cells

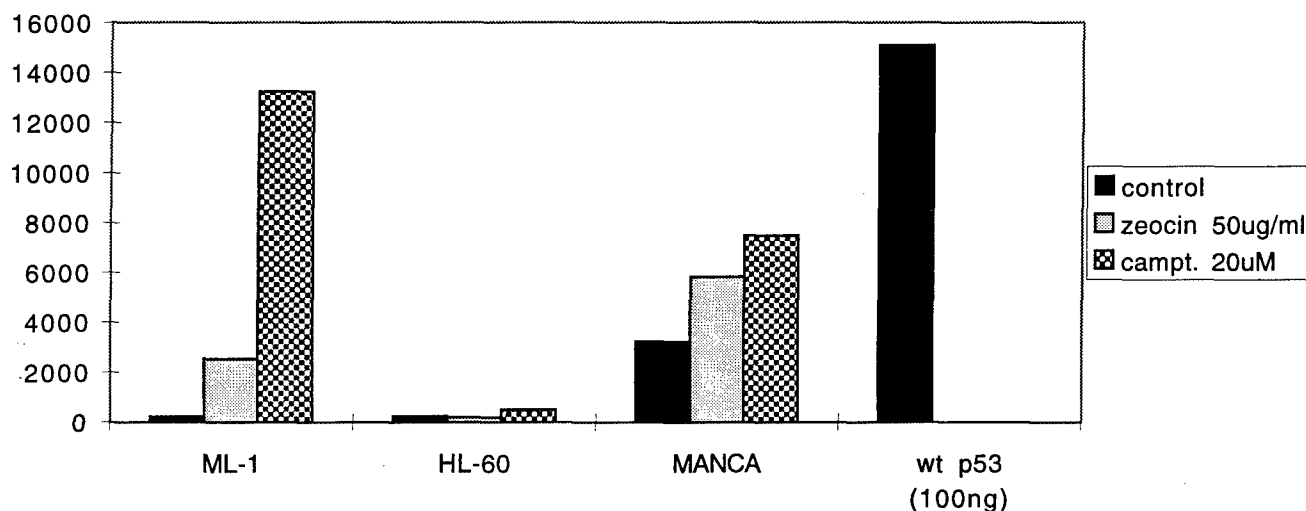


Figure 14 a: p53 protein level in drug treated ML-1, HL-60 and MANCA cells. Cells were incubated for 6 hours untreated (lane1), with Zeocin 50ug/ml (lane2) and Camptothecin 20uM (lane3) and lysed; then 300ug of nuclear protein extract was immunoprecipitated with PAb421 cross-linked to ProteinA-Sepharose beads and analysed by SDS-PAGE Western blot analysis with mixture of PAb1801, PAb421, PAb240.

b: Laser densitometry analysis of film from ECL detection. Film was scanned, using Molecular Dynamics laser scanning densitometer and Image QuaNT (Version 4.1) software and plotted in Excell. The bars reflect pixel density of the scanned film.

**Figure 15a: EMSA with gadd45 oligos
drug treated ML-1 and HL-60 cells**

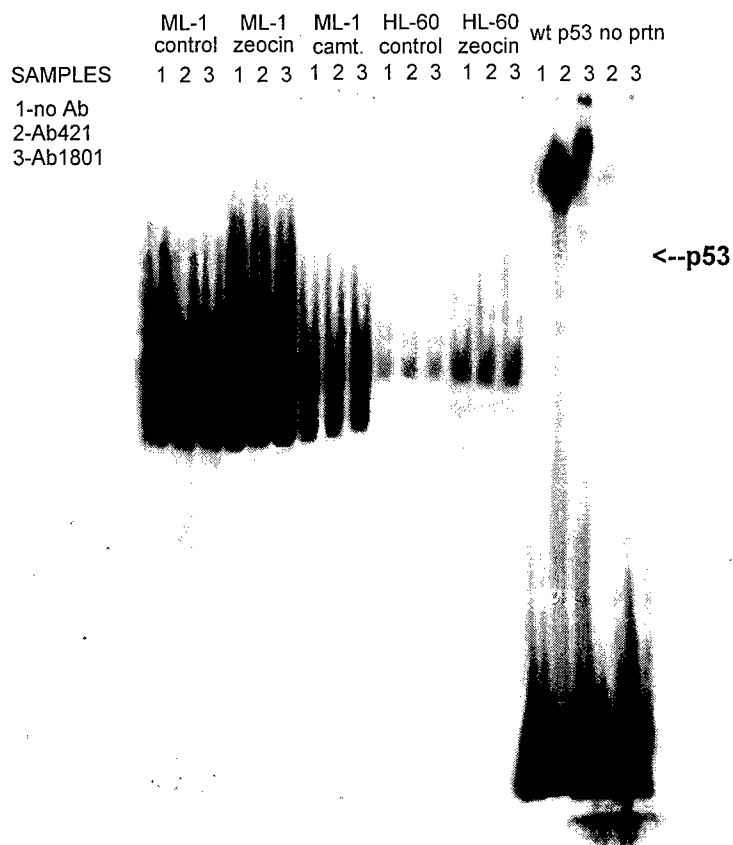


Figure 15a: Electrophoretic Mobility Shift Assay with gadd45 p53-binding site and extracts from drug treated ML-1 and HL-60 cells.

Nuclear extracts from ML-1 and HL-60 cells were isolated 6 hours after incubation without drug or with Zeocin (50mg/ml) or Camptothecin (20uM) and 8 ug were incubated with [³²P] labeled DNA corresponding to the human gadd45 p53-binding site, with or without p53 antibodies (lane 1-no antibodies; lane 2-Pab421; lane 3-Pab1801), then samples were electrophoresed in a neutral 4% acrylamide gel. After autoradiography film was scanned using Molecular Dynamics laser scanning densitometer and analysed with ImageQuaNT (Version 4.1) software.

**Figure 15b: EMSA with mdm2 oligos
drug treated ML-1 and HL-60 cells**

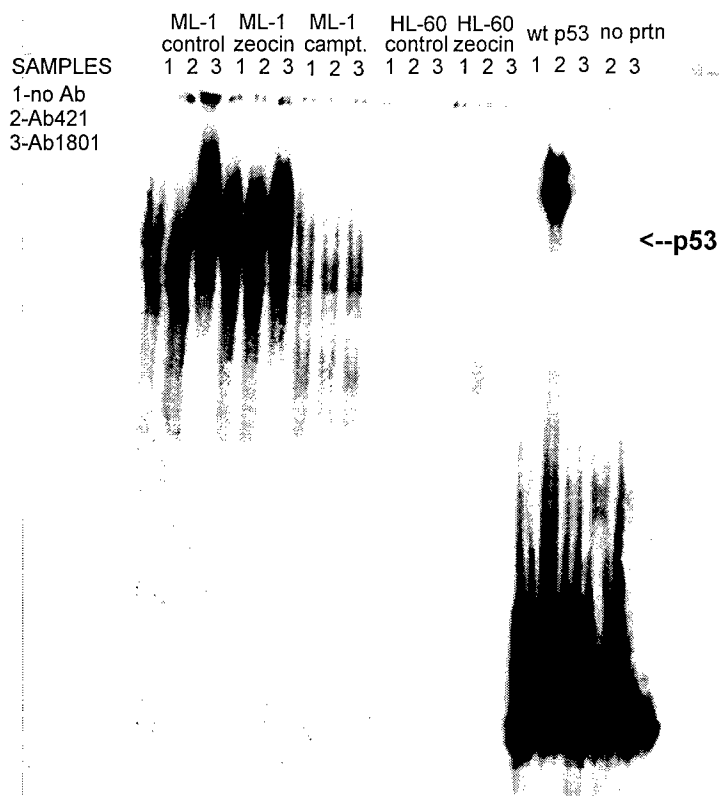


Figure 15b: Electrophoretic Mobility Shift Assay with mdm2 p53-binding site and extracts from drug treated ML-1 and HL-60 cells.

Nuclear extracts from ML-1 and HL-60 cells were isolated 6 hours after incubation without drug or with Zeocin (50mg/ml) or Camptothecin (20uM) and 8 ug were incubated with [³²P] labeled DNA corresponding to the human mdm2 p53-binding site, with or without p53 antibodies (lane 1-no antibodies; lane 2-Pab421; lane 3-Pab1801), then samples were electrophoresed in a neutral 4% acrylamide gel. After autoradiography film was scanned using Molecular Dynamics laser scanning densitometer and analysed with ImageQuaNT (Version 4.1) software.

Figure 16a

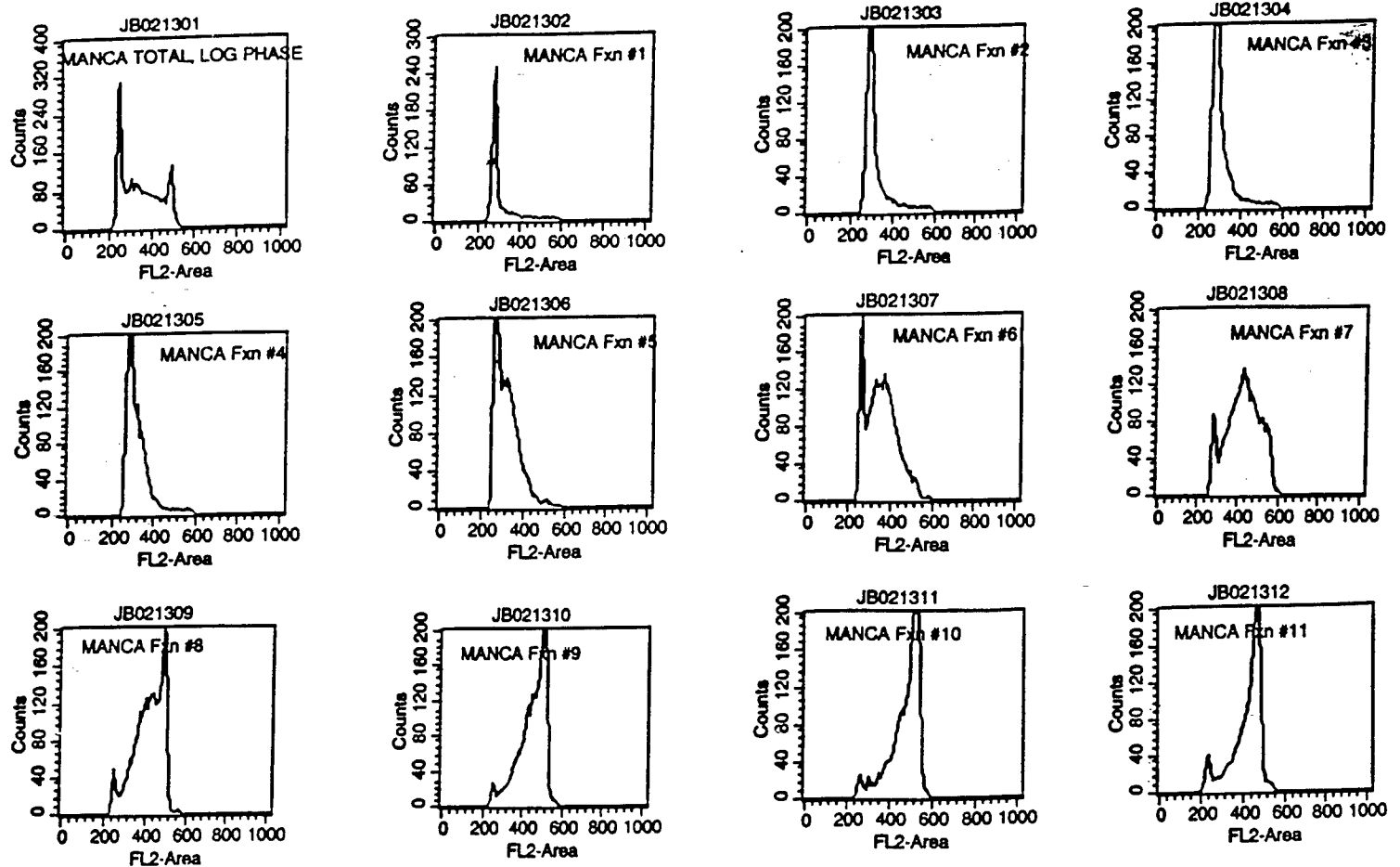


Figure 16b

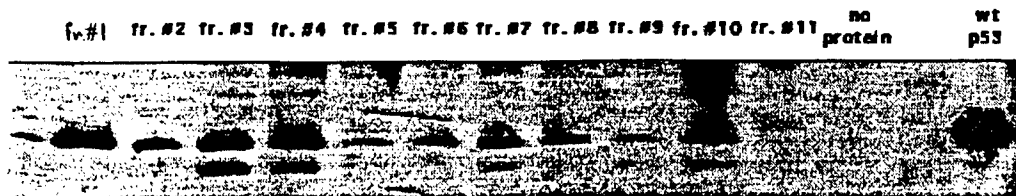


Figure 16c

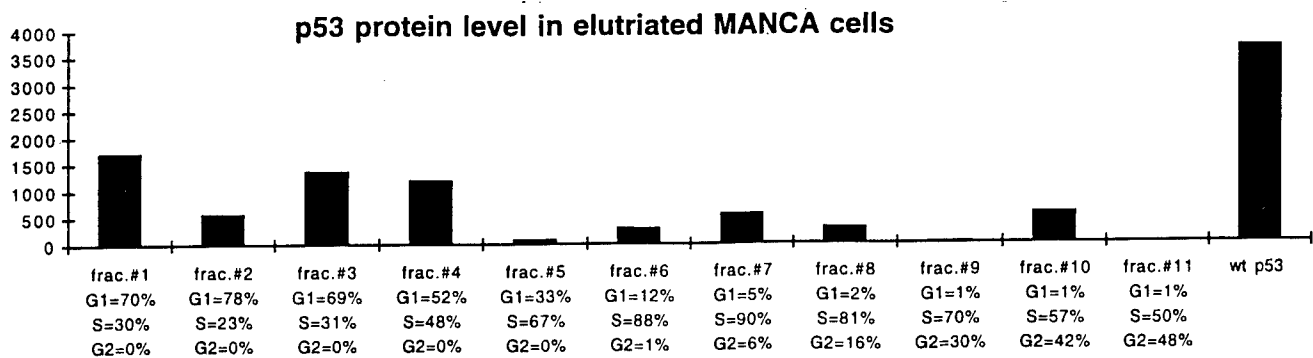


Figure 16: p53 protein levels in centrifugal elutriated MANCA cell fractions.

Four liters of MANCA cells at 4×10^5 cells/ml were elutriated using a Beckman elutriation rotor system.

A) The profile of the elutriated fractions was determined by FACS analysis. Nuclear extracts were prepared and immunoprecipitated as described in the methods. **B)** The p53 present in the immunoprecipitate was resolved by 10% SDS-PAGE and visualized by Western blot with a mixture of p53 specific antibodies PAb421, PAb1801 and PAb240 followed by detection with ECL reagent. **C)** The relative levels of p53 are presented as a histogram of pixel values determined by laser densitometer analysis and quantitation by Image QuANT software (Version 4.1).

Figure 17

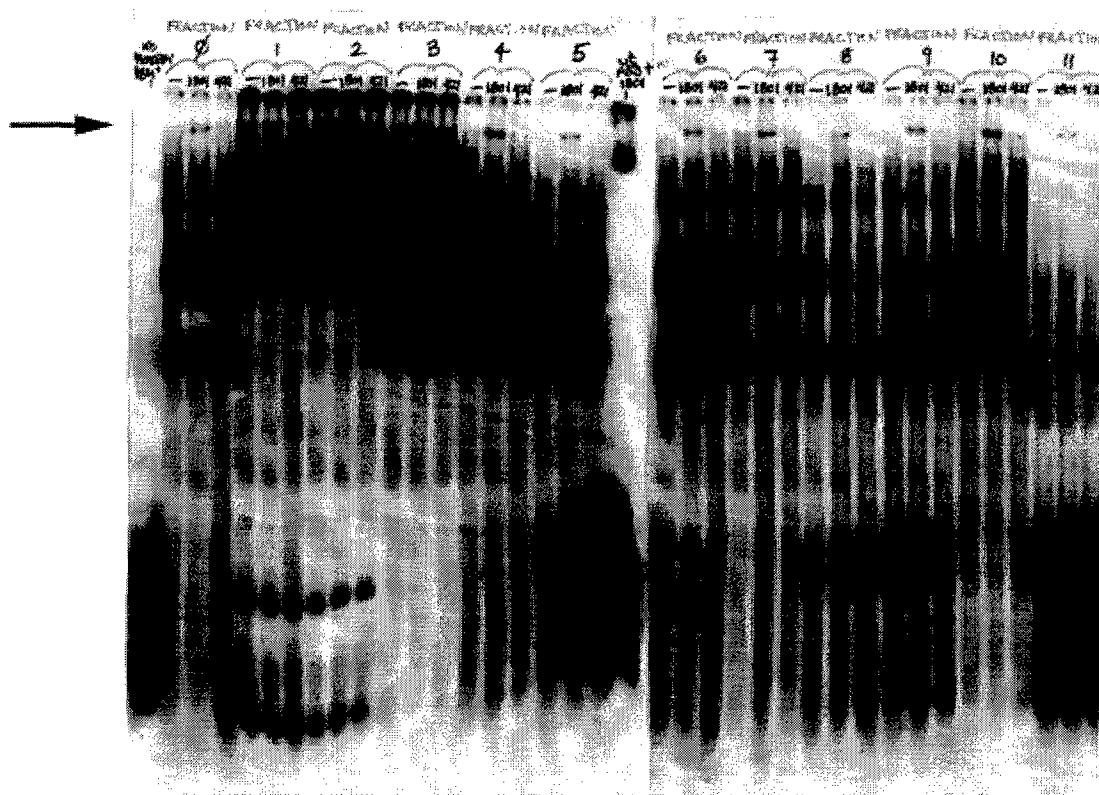


Figure 17: EMSA with nuclear extract from elutriated MANCA cell cycle fractions
 EMSA was carried out as described in the methods. Reactions are presented as three grouped lanes containing no antibody, PAb1801 or PAb421. "Zero" corresponds to unelutriated extract. Groups 1-11 correspond to the elutriated fractions.

Figure 18. RGC, HIV-LTR and MDM2 promoter activity in MDA-MB 468, ZR75.1 and MDA-MB 157 breast cancer cell lines.

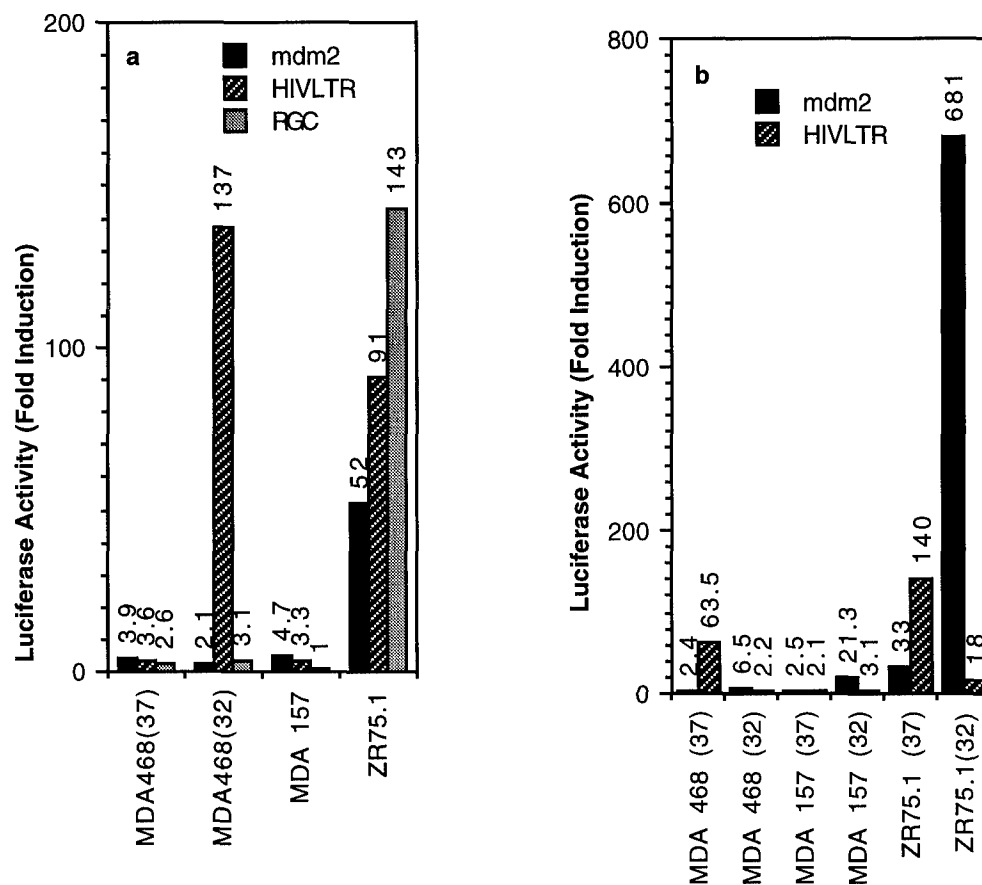


Figure 18: Histogram of fold induction. The percentage fold induction compared to background is indicated above each bar. a) 20µg of supercoiled plasmid DNA was transfected by electroporation into 1×10^7 cells per ml. Cells were grown at 37°C for 48 hours and then either left at 37 or shifted to 32 for 24 hours. b) 10µg of a plasmid encoding the temperature sensitive p53 Val135 was cotransfected with 10µg of the indicated plasmid and treated as above.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information
Center, ATTN: DTIC-OCF, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

<u>Contract Number</u>	<u>Accession Document Number</u>
DAMD17-94-J-4030	ADB215484 ✓
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864 ✓
DAMD17-94-J-4267	ADB216187 ✓
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562 ✓
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management